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CANCER RESEARCH

VOL. 13 DECEMBER 1953 No. 12

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CANCER RESEARCH

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Microbiology and Cancer Therapy: *A Review**

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The use of micro-organisms and their products as possible therapeutic agents in the control of cancer had its beginning in the latter part of the nineteenth century, coinciding with the embryonic achievements of the science of bacteriology. The rapid advances in the field of antibiotics during the past 12 years have inspired new hope that the search among biological systems will disclose a chemical agent which will exert a destructive effect upon neoplastic growth without seriously affecting normal cells.

BACTERIA

The early history of the role of bacteria and bacterial toxins in this development has already been well reviewed (42, 101, 119, 145). Many of the investigations were prompted by the clinical observation that a concurrent bacterial infection frequently retarded the development of malignant processes in man. Some were based on the belief that organisms isolated from neoplastic tissues were the causative agents of cancer (39). Attempts were made to prepare vaccines and serums against such organisms; the efficacy of these preparations is highly doubtful. Although beneficial results were sometimes claimed, attempts to confirm such observations were usually unsuccessful; certainly no cures were effected.

The first record of an attempt to treat human tumors with an induced bacterial infection is that of Busch in 1868 (19). He had observed temporary clinical improvement in two patients with inoperable sarcomas who also developed concurrent erysipelas infections. His attempts to induce the infection in other cancer patients were unsuccessful, because the causative agent of erysipelas was not known at that time. Later, Fehliesen (38), after he had discovered the streptococcal origin of erysipelas, injected live cultures of the bacterium into cancer patients with encouraging results. Since then many varieties of micro-organisms have been reported to be beneficial therapeutic agents. Preparations known as "Coley's Toxins" (100, 101), which have usually consisted of heat-treated mixtures of streptococci and *Serratia marcescens*, have been used clinically for many years, although their effectiveness has not been clearly established.

Beebe and Tracy (13) reported that injections of suspensions of *Bacillus prodigiosus* (*S. marcescens*) cells, either alone or combined with streptococci, resulted in rapid and complete disappearance of transplanted lymphosarcoma in three dogs.

Baroni (8) obtained some measure of protection against the growth of the Jensen tumor in rats if the animals had received injections of streptococcal or gonococcal toxins for several days prior to the implantation of the tumor. Simpson and Marsh (127) were unable to demonstrate any effect, either macroscopic or microscopic, upon the growth of spontaneous mammary adenocarcinoma in mice with tuberculin preparations.

Daels (27) and Comsia (23) found that spirochete infections in mice interfered with the development of tumors. The effect was most pronounced when the animals were infected with the spirochete several days before the tumors were implanted.

Connell (24) reported clinical improvement in several cases of far advanced cancer upon treatment with sterile filtrates of *Clostridium histolyticum*. The filtrates were prepared by cultivation of the bacterium on pieces of human malignant tissue

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suspended in normal saline and were thought, by virtue of the method of preparation, to contain proteolytic enzymes which would preferentially attack the proteins of neoplastic tissue without affecting those of normal tissue. Although Connell mentioned that a carcinoma in mice responded to similar treatment, other investigators (29, 53, 106, 114) in well controlled studies with several animal tumors have been unable to confirm his observations. Procedures such as that employed by Connell have proved to be invaluable for the microbial production and isolation of enzymes possessing specific activities (34). They are based on the idea that when a microbial culture is supplied with a particular organic substance, either as the sole source of carbon or nitrogen or both, the organism, in order to grow, must attack that substance. The digestive process may require the formation of an unusual enzyme system or the production of an excessive amount of an ordinary system. It is highly doubtful, however, that such a method can prove satisfactory when an organism is supplied with such a heterogeneous mass of material as is present in whole tissue of either malignant or normal growth. It would seem probable, therefore, that only if and when a substance which is not present in normal cells is found in malignant tissue and is obtained in a purified state that this technic may offer real promise.

Parker *et al.* (104) injected *Cl. histolyticum* spores directly into a rapidly growing transplanted fibrosarcoma in mice and, with the administration of histolyticus antitoxin, prolonged the life span of some animals for as long as 20 days beyond that of nontreated tumor-bearing mice. Histolyticus toxin injected directly into the tumor caused marked regression of both a sarcoma and a carcinoma; in tissue culture the toxin produced severe damage to sarcoma but not to either carcinoma or normal kidney.

Cohen *et al.* (22) incubated slices of a spontaneous mouse carcinoma, the Brown-Pearce rabbit carcinoma, and their autologous livers in cultures of various bacteria. In general, the bacteria were found to injure both the liver and tumor cells equally well or not at all. Only one organism, *Sporosarcina ureae*, damaged the tumor cells but not the liver cells. The active factor appeared to be intracellular; the cell-free culture medium in which the bacterium had been grown was ineffective.

In vitro technics such as that employed by Cohen have somewhat dubious value in the search for agents which might be useful in the control of cancer. Such methods are frequently defended on the basis that when tissues are tested for their response in isolated systems, possible interfering effects of the whole animal body on the compounds being studied are eliminated. Although this is undoubtedly valuable in certain studies of particular biological systems, the application of any agent to cancer therapy depends to a great extent upon its fate in the living animal. Materials which are active *in vitro* may exert no demonstrable effect *in vivo* (71, 135). Conversely, an agent may cause marked response *in vivo* but be inactive *in vitro* (117). It is not inconceivable that enzyme systems in the body may alter compounds in such a way as to either increase or decrease their biological activities. Some agents may have very low therapeutic indices, and, although they exert pronounced effects upon tumor growth *in vitro*, the dose levels necessary to bring about responses *in vivo* can never be attained.

Gregory, who believes all cancers to be virus-induced (49, 50), has claimed to have seen by electron microscopy *in vitro* destruction of the "cancer virus" caused by *Bacillus subtilis* Tracy 1 (51). By chemical and electrophoretic technics, he obtained from extracts of the bacterium a crystalline material to which he has given the name magnesium tracinate. The crystals were administered to eight patients with far advanced cancer; all were reported to show clinical improvement. The possible merits of tracinate must await more extensive clinical trial, but it would appear to be somewhat presumptuous to attribute any effect that it might have upon neoplastic growth to antiviral properties until more decisive evidence as to the nature of the causative agent or agents of cancer is available.

In 1931 Gratia and Linz (47) reported that *Bacterium coli* filtrates injected into guinea pigs with sarcoma elicited the Shwartzman reaction (125) in the tumor tissue with no observable hemorrhagic lesions in other organs. Motivated by this observation, Shear and his associates began a study of the nature of the hemorrhage-producing agent (122). Shear and Andervont (123) concentrated the factor from *B. coli* filtrates by a method used for the precipitation of the soluble specific polysaccharide from pneumococcus broth cultures; the active fraction was found to give negative biuret and positive Molisch reactions (120).

The group of investigators at the National Cancer Institute later turned their attention to studies on the hemorrhage-producing properties of *Serratia marcescens*. Shear and Turner (124) obtained active preparations which were rich in polysaccharides. Further experimentation confirmed the polysaccharide nature of the material (56) and indicated that its molecular weight is about 8 million (69). Preparations of the purified polysac-

charide were found to produce hemorrhage in mouse tumors in doses of a fraction of a microgram (121). More recently, Ikawa *et al.* (66) have reported the isolation of a hemorrhage-producing agent from filtrates of *E. coli* which they characterized as a complex polysaccharide containing both a peptide and a phospholipid component. Upon treatment of this material with trichloroacetic acid, most of the peptide component could be removed; the lipo-polysaccharide which remained was found to contain most of the original tumor-destroying activity. Thus, it would appear that the peptide portion is not essential for activity.

Brues and Shear (17) administered the polysaccharide from *S. marcescens* to four patients with advanced inoperable malignant tumors. In two of the cases, some relief from symptoms was obtained, and, on post mortem examination, evidence of hemorrhage in the tumors was found. Gross changes in tumor size and consistency as well as microscopic evidence of necrosis and hemorrhage in both animal (30, 31) and human tumors (63, 103, 112) have been reported.

The usefulness of bacterial polysaccharide as a chemotherapeutic agent would appear doubtful. No human tumor has been completely destroyed and rather severe symptoms of toxicity have been encountered (63, 103). Nevertheless, it is to be hoped that studies with the polysaccharide may contribute to the knowledge of the treatment of neoplastic diseases.

Gardner *et al.* (40) produced hemorrhagic reactions in a transplantable rat tumor with extracts of a variety of bacteria including the Neisseriae group, *Klebsiella pneumoniae*, and enteric organisms. Jacobs (67) has reported similar effects on mouse Sarcoma 37 with a polysaccharide-containing fraction from *Pseudomonas aeruginosa*.

Although the role of bacteria in cancer therapy has intrigued the minds of clinician and research investigator alike for several decades, no real insight into the mechanics of the interference of tumor development by bacterial products was available until the observations of Gratia and Linz (47) on the Shwartzman phenomenon in guinea pig tumors aroused new interest. Shwartzman and Michailovsky (126) and Duran-Reynals (35) found that tumors in animals which are not normally susceptible to the Shwartzman phenomenon, namely rats and mice, became hemorrhagic upon systemic administration of bacterial filtrates. On the basis of his observations, Duran-Reynals concluded that, in order to show this reaction, the tumor must be both malignant and rapidly growing; benign tumors and malignant ones exhibiting slow development do not respond. He suggested that the high sensitivity of malignant tissue to bacterial toxins might be a result of an excessive permeability of the newly formed blood vessels (36). That capillary fragility does play a part in this phenomenon would appear most likely. Andervont and Shimkin (3) observed that ascorbic acid inhibited hemorrhage by bacterial filtrates in transplanted tumors, and Eddy *et al.* (37) reported that the area of tumor destruction produced by Shear bacterial polysaccharide in rats deficient in ascorbic acid was 5.3 times greater than that in control animals. Algire *et al.* (1), however, have concluded that the tumor-necrotizing effect is brought about by the low blood pressure which is known to result from injection of the bacterial polysaccharide. They observed that hypotension was accompanied by a reduction in the circulation of blood in the tumor and by subsequent damage to the tumor. Neither circulatory disturbance nor necrosis in the tumor occurred in the absence of hypotension.

Although the literature contains many isolated and conflicting views on the type of bacteria that are capable of causing damage to neoplastic tissue, Zahl and his associates (65, 145, 146), in a survey of a wide range of bacterial species, found that the ability to induce hemorrhage in tumors was with few exceptions a property of gram-negative bacteria, while gram-positive bacteria with the exception of two isolates of Listeria were unable to elicit the response. In view of the many reports that gram-positive bacteria, particularly the erysipelas-producing streptococci, have had therapeutic effects upon tumors, it must be concluded that the mode of any action of such organisms would be quite different from that of the gram-negative bacteria.

FUNGI

Fungi, too, have received a fair share of attention as organisms possessing therapeutic properties against cancer. A brief discussion of some of the early attempts in this field has been given by Fichera (39). Karo (70) claimed to have obtained both subjective and objective improvement in several cases of human cancer as the result of treatment with fermentation products of the mushrooms *Agarius rufus*, *Echinacea*, *Merulius lacrymans*, and *Phallus impudicus* which had been detoxified by extensive ultraviolet irradiation and to which several metallic salts were added. No details of the procedure of preparation were given nor was any mention made of what role the metallic salts themselves might play beyond the statement that they increased the therapeutic effectiveness of the fungal proteins.

McLeod and Ravenel (86), acting on the theory

that if the high fermentative capacity of cancer tissue could be suppressed, the tissue would revert to normal growth, employed fungal preparations as sources of materials thought to be lacking in tumor cells. Although extracts of a variety of fungi were employed, efforts were concentrated on the filamentous fungus *Aspergillus niger* and the yeast *Saccharomyces cerevisiae*. Approximately one hundred and fifty cases of advanced neoplasms considered as hopeless were treated. These investigators stated that practically all the patients experienced subjective improvement, and, in many, notable shrinkage of the tumor masses occurred, although no cures were effected. Upon microscopic examination of biopsy specimens, extreme grades of degeneration and even necrosis were observed. Unfortunately no details concerning the clinical picture or laboratory findings on these patients were given. It is equally distressing that the procedures employed in the preparation of the fungal extracts were not described.

Hulshoff Pol (64) has reported the regression of tumors in mice on a diet containing bread upon which the fungus *Penicillium spinulosum* Thom had been grown.

DeAngelis (28) has claimed very good results against Ehrlich's adenocarcinoma implanted in mice after treatment with a crude culture filtrate of *Streptothrix felis* D.A. which he isolated from a granuloma in a cat. The material, which he named "mycetin," was passed through a bacteriological filter and administered in doses of 0.2–0.4 cc.; the MLD was 0.5 cc. Injections were usually made subcutaneously but at some distance away from the site of the tumor; most animals received only a single dose. Of a total of 330 treated mice bearing tumors ranging in age from 12 to 25 days, tumors regressed in 308 or about 93 per cent of the cases. All 286 control mice died as a result of tumor development.

In 1946 Stock *et al.* (135) initiated a program for the systematic screening of materials of natural origin for their ability to inhibit the development of tumors. Crude culture filtrates of several fungi were found to have a destructive effect upon Sarcoma 180 and mouse melanoma tissue *in vitro* and to cause inhibition of the growth of Sarcoma 180 and of mammary adenocarcinoma E 0771 *in vivo*. Reilly and Stock (110) isolated from the metabolic filtrate and pellicles of some strains of *Aspergillus fumigatus* a protein-like agent having the ability to retard markedly the growth of Sarcoma 180 in mice. The tumor-arresting property was accompanied by a generalized toxicity; efforts to separate the two factors were unsuccessful. Petermann *et al.* (105), in electrophoretic studies, located the active principle in a group of highly basic protein components.

That certain yeasts do have some effect upon neoplastic growth seems probable. Laclau and Imaz (75) reported that hydrolysates of *Saccharomyces cerevisiae* which were biuret-negative had an inhibitory effect upon neoplasms in human beings. Laclau *et al.* (76) reported good results in healing epitheliomas by intramuscular administration of hydrolysates of *Saccharomyces cerevisiae* combined with selenium preparations. The role of the yeast preparation here is open to some question; the authors themselves attribute the effects observed to the formation of dissociation products of selenium.

Nevorojkin (102) found that when a dense suspension of the yeast *Saccharomyces cerevisiae* No. XII (Berlin race) in normal saline was injected directly into the Ehrlich tumor in mice, the growth of the tumor was delayed, and, in some cases, regression occurred. Thin suspensions of the yeast, however, not only failed to depress growth but, apparently, even stimulated it. Maisin *et al.* (87) studied the effect of heat-treated bakers' yeast upon the development of benzpyrene-induced tumors in mice. The yeast, suspended in water, was heated at 80° C. for 6–7 minutes; the mixture was added to a control diet so that each mouse received 0.25 gm. of yeast per day. At the termination of the experiment in the group of 100 control mice there were 51 survivors with an incidence of tumors of 48.1 per cent, of which 23.2 per cent were malignant. At the same time, in the yeast-treated group (100 mice), 60 were still living. These had an incidence of all tumors of 28.3 per cent and an incidence of cancer of only 1.2 per cent.

In *in vitro* experiments Protti (108, 109) observed that tumor tissues immersed in liquid cultures of a large variety of yeasts and incubated at 30° C. for 48 hours or at 37° C. for a shorter period of time showed marked lysis; normal tissues were unaffected. A selective action was noted; certain varieties of yeasts affected some of the tumors but not others.

Lewisohn and his associates (83) reported that an aqueous extract of brewers' yeast caused regression of spontaneous mammary adenocarcinoma in mice. Of 33 animals treated, tumors disappeared in eight and were reduced in size in another ten; in fifteen mice either there was no change or the tumor increased in size. Further investigation (85) disclosed that the reported active principle was water-soluble, fairly stable to heat at neutral pH, and was not a protein. None of the known B vitamins, including thiamine, riboflavin, pyridoxine,

nicotinic acid, pantothenic acid, and *p*-aminobenzoic acid, appeared to be responsible for the antitumor property (77, 84), although the addition of either pantothenic acid or riboflavin to an active yeast extract appeared to improve the effectiveness of the latter in prevention of the development of carcinoma 2163 in the RIII strain of mice. Later, inositol (78) and "*L. casei* factor" or folic acid (79, 80) were reported to be relatively potent inhibitors of tumor growth. Sugiura (136), however, was unable to confirm the inhibition of spontaneous mammary adenocarcinoma by yeast extracts or folic acid, although he made a special effort to follow the procedure of Leuchtenberger and associates. Indeed it is difficult to understand the highly favorable results reported with folic acid, since it would appear that certain of the folic acid antagonists have a pronounced deleterious effect upon neoplasms (134).

Laszlo and Leuchtenberger (77) found that some lots of brewers' yeast did not contain the antitumor factor. The deficiency was attributed to a change in the conditions under which the yeast was grown. While this explanation seems reasonable, the earlier observations of Heaton (60) on this problem cannot be disregarded. This investigator found that extracts of brewers' yeast contained an agent which inhibited both normal and tumor growth. His observation that different samples of the yeast varied in activity, coupled with the fact that extracts of bakers' yeast did not cause the effect, led him to think that the active principle was not a product of the yeast but was a constituent of the medium on which the organism had been grown. Further experimentation indicated that malt, a constituent used in the cultivation of brewers' yeast, was actually the source of the growth-arresting agent; results similar to those seen with the active yeast extracts were obtained with commercial malt extract.

Protozoa

Roskin and Klyueva (72, 113) found that infection with the protozoan *Trypanosoma cruzi* caused regression of tumors in mice. As a result of more than 15 years of investigation they claimed to have separated from cells of *T. cruzi* an agent having the ability to destroy malignant neoplasms in man as well as experimental tumors in mice. Although Malisoff (88) reported confirmation of these findings in his work with two mouse tumors, a spontaneous mammary carcinoma and transplanted Sarcoma 180, a number of other investigators (57) have been unable to prepare active *T. cruzi* lysates. Hauschka *et al.* (59), in excellent studies, were able to retard significantly the growth of three transplanted mouse tumors by active infection of the animals with *T. cruzi*, but preparations of endotoxin (heat-killed cells) were without effect. These results were open to some question, because the work had been done with a strain of *T. cruzi* which was different from that used by the Russian workers. Hauschka and Goodwin (58) later extended the investigations, including in their studies five types of mouse tumors, both implanted and spontaneous, and eight different strains of *T. cruzi*, including the strain used by Malisoff and the one which was, according to Roskin and Klyueva, the source of the K-R factor. Again, they were unable to obtain a tumor-destroying preparation.

In 1948 Gruhzit and Fisken (52) reported failure to obtain any effect upon the development of the Brown-Pearce carcinoma in the rabbit with a lysate of *T. cruzi*. Jedeloo *et al.* (68) were unable to demonstrate any inhibition of the development and subsequent growth of a tar-induced epidermoid carcinoma in mice with endotoxin preparations of *T. cruzi*. Spain *et al.* (132) obtained negative results with whole culture lysates of *T. cruzi* in the treatment of mammary carcinoma and transplanted Carcinoma 119 in mice.

Nadel and Greenberg (99) have reported an increase in survival time in leukemic mice when *Plasmodium bergei*, the infectious agent of malaria, was inoculated into animals after leukemia had developed.

Antibiotics

For the purpose of this discussion antibiotics will be considered as defined by Waksman (144): "An antibiotic is a chemical substance, produced by microorganisms, which has the capacity to inhibit the growth and even destroy bacteria and other microorganisms, in dilute solutions."

Although Boyland (16) found that some aromatic sulfur compounds which have antibacterial properties retarded the growth of spontaneous mammary tumors in mice, there is no a priori reason to expect antimicrobial agents to be effective against tumors. Nevertheless, the highly specific nature of the action of antibiotics (143), that is, the ability of such substances to inhibit the growth of one micro-organism without affecting another closely related one, even within the same species, indicates the possibility that some of them might display a similar selection between normal and neoplastic cells. Certainly, there is evidence that, under special conditions, such may be the case.

Over 40 years ago, Uhlenhuth *et al.* (141) and Beck (12) reported that pyocyanase, an antibacterial agent now generally considered as the first antibiotic to be isolated, caused regressions when

injected directly into tumors implanted in mice and rats. Unfortunately, as pyocyanase is a rather toxic agent, this line of investigation did not progress very far.

With the advent of penicillin, an essentially nontoxic chemical of remarkable therapeutic benefit in the control of certain infectious diseases, it was only natural that the attention of those interested in the control of cancer should be turned in this direction. Cornman (25, 26) observed that in tissue culture crude penicillin preparations exerted a lethal action upon cells of mouse and rat tumors at concentrations that did not damage normal cells. Although she confirmed Cornman's result with crude material, Lewis (82) found that highly purified penicillin had no effect upon tumor growth in tissue culture. Other investigators (43, 91, 135) have reported similar experiences.

Beard (11) has reported that the administration of crude penicillin to rats bearing implanted Emge sarcoma apparently reduced the number of takes and caused regression of a high percentage of tumors. In three experiments with a total of 82 rats treated with penicillin, disappearance of the tumor occurred in 35–72 per cent of the animals, while regressions in 148 untreated control rats was less than 20 per cent. Dobrovolskaia-Zavadskaia (32) has obtained histological evidence that yellow sodium penicillin does interfere with the growth of tumors in mice; the outstanding characteristic was the development of a massive hyperemia in the neoplastic tissue. The effect was temporary; when treatment was discontinued, the tumor reestablished itself. Those areas which had been subjected to treatment, however, appeared to be permanently damaged. In one patient with mammary adenocarcinoma, she (33) saw marked clinical improvement under penicillin therapy and observed histological changes similar to those seen in mice. Bennison (14), on the other hand, could find no difference in the incidence of the development of mammary tumors in mice carrying the milk factor upon treatment with impure penicillin. Stock (133) has reported that even at a dose of 5 gm/kg body weight/day crystalline penicillin G had no effect upon the growth of Sarcoma 180 *in vivo*.

It is of interest that Burk *et al.* (18), in studies of effects on the metabolism of tissues, observed that several preparations of amorphous penicillin, varying in potency from 1,000 to 1,500 Oxford units/mg, brought about a marked inhibition in the respiration of several tumor and normal mouse tissues, while crystalline penicillin G (1,660 Oxford units/mg) was only one-tenth as active on a weight basis. Burk considered it possible that the slight activity shown by the highly purified material might be the result of the presence of a small amount of impurity.

Levine *et al.* (81) isolated from culture filtrates of *Penicillium notatum* a nonpenicillin-containing material which they called penichromin. In Warburg studies with mouse liver homogenates penichromin inhibited the oxidative utilization of several intermediary metabolites but did not affect succinoxidase, cytochrome oxidase, or anaerobic glycolysis. *In vivo*, the preparation had no effect upon the growth of an adenocarcinoma or a lipoma in mice. These investigators concluded that "in view of the *in vitro* data showing partial inhibition, the failure of penichromin alone to produce tumor regression was not unexpected." This conclusion would not appear to be fully justifiable on the basis of the evidence presented. Treatment in mice was not begun until about 1 week after implantation of the tumors, and only one dose level, 2 mg/mouse daily for 5 days, was used. No data were presented to indicate the relationship of this dose to the maximum tolerated dose. It is possible that the utilization of younger tumors and higher doses of penichromin might have given more promising results.

In view of the few encouraging reports on the effects of crude penicillin on tumors and observations in our own laboratories of quite definite but elusive inhibitory effects of crude culture filtrates of *P. notatum*, it seems probable that a more thorough and systematic investigation of this problem might prove fruitful.

Notatin, a second antibacterial agent produced by *Penicillium notatum*, was chosen by Carr (20) as being of possible interest in the control of the Rous sarcoma virus. He reasoned that it might be effective, because the action of notatin has been shown to be the result of the production of H_2O_2 in the oxidation of glucose to gluconic acid (115) and because, according to Carr's interpretation of the data of Gye and Purdy (54), the Rous virus is readily destroyed by oxidation. This second reason would appear to be erroneous because, as Gye and Purdy pointed out, their experimental evidence does not warrant a decision as to whether the loss of potency of the Rous virus was the result of oxidation or was brought about by destructive proteolytic enzymes. Regardless of the possible error in his hypothesis, Carr did find that, *in vitro*, complete loss of virus activity could be accomplished when the virus was mixed with a solution containing notatin and glucose. When, however, notatin was injected into fowls either before inoculation with virus or after development of tumors, no effect upon either the incidence of infection or growth of the tumors was observed. Chinn (21) re-

ported similar *in vitro* effects upon the Rous virus with terramycin, aureomycin, neomycin, and antibiotic PA96 (Pfizer). When, however, chickens, previously infected with virus, were treated with aureomycin or terramycin, the course of the disease was not altered. In fact, with low concentrations of the antibiotics there appeared to be a stimulatory effect upon tumor development.

Stock *et al.* (135) found that immersion of the tissue in a solution of clavacin (1 mg/cc) completely destroyed the viability of Sarcoma 180 and a mouse melanoma, while streptomycin (10 mg/cc) appeared to have no effect. Gliotoxin, in an aqueous suspension of 1 mg/cc, caused an 80 per cent reduction in the growth of treated Sarcoma 180 implants but had no effect upon the melanoma. Stock (133) later reported that clavacin and gliotoxin had only very slight, if any, effect upon the development of Sarcoma 180 *in vivo*.

Kidd (71) found that cells of two mouse tumors and of the Brown-Pearce rabbit carcinoma were rendered nonviable by immersion for a short time in crude culture filtrates of the fungus *Aspergillus fumigatus*. As a result of comparison experiments, he indicated that the active principle in his fungal filtrates was possibly identical with gliotoxin. He was unable to demonstrate any effect with potent culture filtrates upon the development of the Gardner lymphosarcoma in mice. Mason and Kidd (90) concluded that the cyclic disulfide linkage which is present in gliotoxin is necessary for the destruction of tumor cells *in vitro*.

Vollmar (142) observed that malignant cells in tissue culture were inhibited by patulin (clavacin) at concentrations that not only did not damage normal tissue but actually stimulated its growth, a factor which might be of interest in wound healing.

Barnard and his associates (6, 7) reported that, when the usual course of therapy yielded no benefit to two patients with leukemia, the oral administration of a crude fermentation concentrate, which was obtained from Chas. Pfizer & Co., resulted in both clinical and hematological response. This was at first attributed to the high concentration of vitamin B_{12} in the concentrate, but further investigation caused Barnard to conclude that not B_{12} but rather any of the streptomyces-derived antibiotics (terramycin, aureomycin, streptomycin, or chloramphenicol) could bring about temporary remission in leukemia (5, 7). Goldman (46) observed no alteration in the clinical course of the disease in five patients with Hodgkin's disease upon the administration of aureomycin. Ayres (4) treated thirteen cases of what he termed "anaplastic lesions (carcinoma *in situ*) of the cervix" with topical applications of aureomycin. He claimed to have obtained complete regressions in six of these patients. The diagnoses of these cases are open to severe question, and the malignant nature of the lesions is highly doubtful. It is most probable that any lesions which may have responded to aureomycin therapy were infectious, not cancerous, in nature. Further, it is significant that, in five cases of advanced carcinoma, Ayres noted no real beneficial effect upon the administration of aureomycin.

Bateman *et al.* (10) employed aureomycin as an adjuvant in the treatment of cancer patients who were receiving x-ray and massive HN2 therapy. Administration of the antibiotic was followed by gross and nonspecific microscopic effects on the tumors and in some cases appeared to potentiate the effects of x-ray and HN2. Five cases which had previously been considered as inoperable were modified sufficiently to permit surgical treatment.

Malmgren and Law (89) found that aureomycin, chloramphenicol, and streptomycin did not affect the tumor-producing properties of the mammary tumor milk agent in C3H mice. When the milk agent was injected into strain C mice, the data suggested that streptomycin may have activated the virus; the mean tumor age in the antibiotic-treated group was somewhat lower than that in the control animals. Ambrus *et al.* (2) have found that aureomycin, terramycin, and chloramphenicol have no inhibitory effects upon the development of several tumor-inducing viruses either *in vivo* or *in vitro*.

Sokoloff and Eddy (128) observed a very definite stimulation of implanted carcinoma in rats fed a small amount of aureomycin (1.6 mg/day/100 gm body weight for about 1 week). Large doses (8 mg/day/100 gm), however, caused a marked inhibition of tumor growth as well as severe loss in total body weight.

Stock (133) reported that of eleven antibiotics tested only two, actinomycin and citrinin, caused reproducible slight inhibitory effects on the growth of Sarcoma 180 in mice. Reilly *et al.* (111), in a survey of 33 antibiotics, found that only five—actinomycin, actidione, illudin M, illudin S, and terramycin—caused a slight retardation of the development of Sarcoma 180 *in vivo*.

Hackmann (55) found that, when suspensions of the Ehrlich ascites carcinoma, mouse Sarcoma 37, or the Walker rat carcinoma were mixed and incubated for a short time with actinomycin C, the viability of the tumors was severely damaged. *In vivo* the antibiotic retarded slightly the growth of the Walker rat carcinoma at dose levels which caused little or no toxicity in the host. Schulte (116) has reported preliminary results on 150 patients treated with actinomycin C, either alone or

in combination with x-ray therapy. Although little response was observed in cases of carcinoma, the effects noted in over 50 patients with lymphogranulomatosis were reported to be encouraging. Doses of 50–250μg. of antibiotic were administered daily over a period of several weeks without harmful effects to the patients. These gained weight and showed an improved blood picture. External tumors disappeared rapidly; mediastinal tumors, however, showed only slight regression. As Schulte has emphasized, conclusions on the merits of actinomycin C as a therapeutic agent against cancer cannot be made at this time. His report comprises results obtained over a period of only 1½ years; in some cases the dose of antibiotic was probably too small. Tests on more patients at higher dose levels as well as post-treatment observations over a longer period of time will be necessary before actinomycin C may be properly evaluated.

Bateman and Klopp (9) have claimed that the concurrent administration of actidione and aminopterin significantly prolonged life in leukemic mice. Their enthusiastic conclusion, however, is not supported by convincing data; the survival of the treated mice was only very slightly greater than that of the untreated animals.

It would appear that any favorable responses which have been observed in the treatment of human cancer with clinically available antibiotics have been the result, not of the ability of such agents to destroy tumor cells, but of their capacity to improve the general condition of the patients temporarily by the control of certain secondary microbial infections. Nevertheless, the suggestive results obtained in laboratory animals with some antibiotics indicate the necessity for a continued search for a cancer-controlling agent among substances of this type.

Viruses

Although a complete analysis of the intricacies of virus interference phenomena is beyond the scope of this presentation, the potentialities of viruses as useful agents in the control of cancer merit some attention. Turner and Mulliken (138) and Moore (92) have reviewed briefly the historical background of this field, and only some of the more recent contributions will be considered here. Turner and Mulliken (138) found that, when brain-adapted vaccinia virus was injected into mice with Sarcoma 180, the virus localized in the tumor tissue. In early studies, infected tumors appeared to grow more slowly and to regress more frequently than untreated tumors. Later, however, these investigators concluded that the virus did not influence the growth of Sarcoma 180 appreciably (139). Moore (92) reported similar results with the viruses of influenza A and herpes simplex. Turner *et al.* (140) obtained some increase in survival time in leukemic mice treated with vaccinia virus, but the slight effect was lost when repeated passage of the leukemia rendered it more virulent (139).

Moore (93) demonstrated definite destruction of Sarcoma 180 in mice which had been infected with the virus of Russian Far East encephalitis. The tumor contained a high concentration of virus and failed to grow when transplanted to mice immunized against the virus. Examination of histological sections showed massive cellular damage. Further investigation disclosed that five other transplantable mouse tumors responded to the Russian virus in a similar manner (94). Koprowski and Norton (73) confirmed Moore's observations with the Russian encephalitis virus and showed further that certain other neurotropic viruses possess oncolytic properties. Toolan and Moore (137) reported that Egypt virus 101 had a destructive effect upon a human epidermoid carcinoma grown in x-radiated rats. Southam *et al.* (129) found that infections with West Nile and Ilheus viruses temporarily inhibited leukemic leukocytosis and infiltration in mice but did not cause any significant increase in survival time. The ability of neurotropic viruses to attack tumors would appear to be specific in nature; each virus has its own "tumor spectrum" (73, 96), a term coined to indicate that all oncolytic viruses do not destroy all tumors but that each virus is effective against only a particular group of tumors which vary with the virus employed.

Unfortunately, the tumor-necrotizing effects of the neurotropic viruses in mice have been achieved at dose levels of the viruses which cause eventual death of the animals. If active infection does not take place, the tumor is not affected (73, 93). Ordinarily, susceptible tumors when grown in mice immunized against the virus do not respond to virus therapy (98). Kuwata (74) has reported similar findings with the ornithosis virus and a mouse carcinoma. Such results present a very dim prospect for the practical application of viruses to cancer therapy. Nevertheless, the observations of several investigators offer some hope that the tumor-destroying properties of certain viruses need not necessarily be accompanied by destruction of nervous tissue. Sharpless *et al.* (118) found that the viruses of Russian and West Nile encephalitis could cause regression of a malignant lymphoid tumor in chickens without killing the host. Ginder and Friedewald (44) have reported rapid necrosis

of rabbit fibroma and some effect upon the development of myxoma in rabbits (45) by the Semliki Forest virus without causing death or apparent illness in the animals. Moore (97) has obtained destruction of Sarcoma 180 by the Russian encephalitis virus without killing the host when the tumor was implanted in PRI strain mice.

Moore (95) has attempted modification of the Russian encephalitis virus to increase its oncolytic properties and simultaneously to lower its affinity for nervous tissue. By repeated tumor to tumor passage of the virus she was able to obtain a strain of virus which destroyed Sarcoma 180 cells more rapidly than did the original strain, but its neurotropic properties remained unchanged.

Kuwata (74) has reported that *Rickettsia tsutsugamushi* and the ornithosis virus multiplied and persisted in two mouse tumors for several days. Although the infections appeared to have no effect upon the development of the original tumors, on bioassay heavily infected tumors showed loss of viability.

In spite of the apparently overwhelming obstacles that are involved in the use of viruses in the treatment of cancer (131), some cautious clinical studies have been made. Bierman *et al.* (15) reported some response in leukemia upon treatment with the virus of feline agranulocytosis. Southam and Moore (130, 131) have made very careful studies of the effects of several neurotropic viruses on the course of human neoplastic diseases. No permanent beneficial effects were achieved, but in a group of 34 patients treated with Egypt virus some temporary objective regression appeared in four, and suggestive but not conclusive beneficial effects were noted in five others. Higgins and Pack (61, 62) inoculated rabies vaccine into 30 patients with malignant melanomas. In at least six of the cases dermal metastases decreased in size and became flattened. The development of new metastases appeared to be retarded.

Although the practical application of viruses in cancer therapy thus far has yielded no outstanding results, experimental evidence indicates that the success of such treatment is not beyond the realm of possibility. A virus that showed a propensity for tumor tissue but was nonpathogenic or only mildly virulent for human beings might achieve results in the clinic similar to those which have been observed with chickens and rabbits. Also, if an antibiotic that was effective against an oncolytic virus were available, it might be possible to infect individuals with the particular virus, and, after a lapse of time sufficient to insure destruction of tumor tissue, to control any ensuing systemic viral infection with the antibiotic. The potentialities of the therapeutic use of viruses have not yet been exhausted and certainly deserve further consideration.

The mechanism of the action of the inhibition of tumor growth by viruses is not known, and, in view of the lack of factual knowledge on this subject, any attempt to explain this phenomenon can be only speculative. Clinical evidence indicates that it is not merely the result of nonspecific factors such as fever or stress (131). Although there is no experimental evidence to support such a theory, several investigators (94, 131, 138) postulate that the oncolytic effects of viruses may be brought about through competitive metabolic antagonisms. Any conclusions on this problem must await further data on the metabolism of both viruses and mammalian cells.

DISCUSSION

Micro-organisms offer an almost infinite source of new and interesting chemical compounds: many have the ability to form substances the very existence of which is beyond the wildest fantasies of man's imagination. In many instances even when the structure of a new microbial product can be elucidated, all efforts to manufacture it synthetically meet with defeat. Thus, it is not unreasonable that the search for an agent which will destroy neoplastic growth should be made among products of microbial metabolism. Such investigations, however, should be undertaken only when the complexities of the problem have been carefully considered and appreciated. The microbiological aspects alone appear quite formidable. As has been clearly pointed out in antibiotic research (143) and, to a lesser extent, even in cancer investigation (108, 109), the ability to produce a specific agent is a characteristic, not of a genus or species, but of a particular strain of micro-organism. In our own experience with seven different isolates of *Aspergillus fumigatus*, five were found to produce tumor-retarding filtrates, while two were completely inactive.

The now widely recognized variability of microbial cultures presents an added hazard. Some organisms, freshly isolated from natural sources, retain most of their original properties on repeated transfer on laboratory media; others very quickly lose their abilities to carry out the special functions for which they were isolated. To keep a culture in an active state, with respect to its capacity to produce a particular substance, requires great care and constant vigilance, which all too frequently is of no avail.

The nutritional and environmental conditions of cultivation markedly influence the metabolic

functions of an organism. Conditions that are optimum for maximum growth may not be suitable for the formation of a specific substance. Frequently an unknown precursor must be supplied, or a proper balance among certain mineral elements must be achieved. Oxygen tension, pH, temperature, and time of incubation must be carefully controlled. Although the number of variables seems most forbidding, the tremendous economic, industrial, and medical importance already achieved in the field of microbial fermentations (107) clearly demonstrates that they are not insurmountable obstacles.

In any attempt to find among natural sources agents that have specific properties either of an enzymatic, antimicrobial, or antitumor nature, one is confronted with the problem of whether highly purified preparations or crude mixtures, such as microbial culture filtrates, should be tested. Both offer definite advantages. Purified agents, even when their chemical structures are not known, are usually well characterized, and preparations from different batches, depending upon their degree of purity, do not vary greatly. Thus, any effects that they may exhibit can be more or less readily reproduced. On the other hand, most pure compounds that are biologically interesting have been isolated because they affect systems other than neoplasms, and, in the process of purification, any component that might possibly have been active against tumor growth has been lost. Such would appear to have occurred in the crystallization of penicillin.

Because they are heterogeneous mixtures, crude materials offer great possibilities. It is well recognized in the field of antibiotics that one microorganism may produce several antimicrobial agents simultaneously; this might well be true of the production of antitumor substances. The possibilities of additive or synergistic effects among several components in crude preparations certainly cannot be disregarded.

In any search for an agent that will be useful in the therapy of human cancer, a most disturbing element is the choice of test procedure and of test tumor. The tremendous strides in antibiotic research and development have been made possible to a great extent by the fact that preliminary tests for demonstrating antimicrobial activity and for following the production and chemical isolation of the active principle can be carried out *in vitro*. In cancer research, although *in vitro* technics do provide excellent tools for detailed studies of specific problems, their use as test procedures to pick up agents which must finally interfere with abnormal growth *in vivo* has not been very fruitful. Thus, it would appear that at present screening for antitumor agents must be conducted *in vivo*. This immediately excludes the possibility of the use of a really rapid and simple procedure.

In view of our scant knowledge, the choice of a test tumor must necessarily be arbitrary. Whether it should be a spontaneous or an implanted one has been a point of contention for many years. There can be no doubt that certain properties of transplanted tumors are different from those of spontaneous ones, even when the former have been derived from the latter (48). Nevertheless, transplanted tumors would appear to be the ones of choice for use in large scale testing programs because they are readily available and offer much greater opportunities for well controlled experimentation than do spontaneous developments.

The fact that different animal tumors have been shown to exhibit varied responses to treatment with chemical agents (41, 133) presents an additional problem. Some tumors may be markedly affected by a particular compound, whereas others will possess a strong natural resistance to it. Thus, any results that may be obtained with one type of tumor provide little or no information as to what may be expected with other tumors.

When all the factors that are involved in this field of investigation are considered, the need for patient and persevering endeavor becomes apparent.

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Antigenic Properties of Methylcholanthrene-induced Tumors in Mice of the Strain of Origin

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Immunity has been produced against transplantable tumors which arose in inbred mice by Gross (3) and in inbred rats by Lewis *et al.* (6, 7). These results have been explained on the basis either that the tumor has mutated during repeated transplantation, or that immunogenetic differences among the inbred mice have developed (4, 8). In the experiments of Lewis *et al.*, immunity to uniformly progressive tumors of inbred rats developed following autolysis of the tumor (6, 7). Fardon and Prince (1), using this technic, failed to demonstrate immunity against spontaneous mammary carcinoma dbr/B in DBA/1 mice, and we (2) have failed to demonstrate immunity against spontaneous mammary carcinoma of C3H-He mice following ligation of the tumor. We have continued these studies with spontaneous mammary carcinoma and with sarcomas induced by methylcholanthrene in C3H-He mice and have obtained results which indicated that these two types of tumors differ in their capacity to alter the susceptibility of mice to reimplantation after prior ligation.

MATERIALS AND METHODS

All experiments were done with C3H (Heston subline) mice obtained from the Jackson Memorial Laboratory. Young adults of both sexes were used. Four of the sarcomas (S215, S290-C1, S290-C2, S299-B2) arose 60–90 days following subcutaneous injection of 1 mg. of methylcholanthrene dissolved in 0.05 ml. of lard, and two (S303, S339) following subcutaneous implantation of a methylcholanthrene crystal. At the time when they were transplanted to new mice, they measured 1.0× 1.0×0.8 cm. Several of the tumors grew slowly on the first transplantation in C3H-He mice, but on subsequent transplantation all grew rapidly, reaching palpable size in 7 days and killing the mice in about 6–8 weeks. Histologically, the tumors appeared as fibrosarcomas with numerous mitotic figures. Routine passages were made in C3H-He mice, and in every instance the tumors grew progressively. Three mammary carcinomas which arose in breeding females were also studied (AC 304, AC 304-1 and AC 322). All implantations were made with a 13-gauge trocar subcutaneously in the region of the right scapula. The tumor fragments introduced weighed 10–12 mg. All mice were palpated at least once a week to determine growth of the tumor and were observed for a period of 2 months following the last challenge implantation. When the transplanted tumors reached 0.75–1.0 cm. in the largest diameter, they were ligated. This was accomplished in lightly etherized mice by firmly, but not tightly, drawing a loop of surgical thread under the base of the tumor made accessible by stretching the skin and tumor outward from the muscles of the back. Twenty-four hours later, a tighter loop was placed around the skin pocket in which the tumor was enclosed. Tumors thus ligated usually dried up within a few days and disappeared leaving a well healed scar. Occasionally, additional loops were necessary on the 3d or 4th day to complete the process. When the tumor invaded the muscle tissue, as occasionally happened, it was not possible to stop its progressive growth. Mice were challenged for immunity by reimplantation of tumor on the opposite flank, at different intervals, 6 or 2 days before, on the same day, or 2–24 days following the beginning of the ligation procedure.

EXPERIMENTAL

Tumor tissue for transplantation and histological study was removed from the mouse in which it arose, by biopsy or after killing the animal, and was transplanted into groups of C3H-He mice. All developed progressive tumors, but, as was the case with tumors S215, S303, S339, and S299-B2, the rate of growth in the first transplant generation varied in the implanted mice. For this reason, most of the experiments were done with a tumor of the second or later generations in which the transplants grew at a more even rate in the recipient mice. The progressively growing tumors were

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ligated when they had reached sufficient size, and the mice in which the tumors disappeared were challenged by reimplantation of tumor fragments. The results are shown in Table 1.

It is seen (Table 1) that C3H-He mice in which Sarcoma 215 was caused to regress as a result of ligation were immune when the tumor was reimplanted. This was observed in mice carrying tumors of all transplant generations at which tests were made. Immunity of the mice was manifest by failure of the implanted tumor to grow at all or by regression of tiny hard palpable nodules. Regression occurred in eleven of the nineteen mice carrying the second transplant generation of tumor S215. The mice found to be immune in the first experiment were reimplanted after 1 month with tumor from the fifth passage generation; all failed to develop tumors. The results with the other methylcholanthrene-induced tumors are of a similar nature; significant numbers of mice in which tumors were ligated were immune when reimplanted with the same tumor. The four mice shown to be immune to S290-C2 were rechallenged 1 month after the first test with tumor of the first transplant generation and again failed to develop tumors, while five normal control mice died of progressive tumors. Ten mice shown to be immune to S215 were challenged with a spontaneous mammary carcinoma CA 108 which arose in a C3H-He breeding female (2). This tumor grew progressively and killed all the sarcoma-immune mice. Similar results have also been obtained with other mammary tumors implanted into mice immune to Sarcoma 339, 290-C1 and 290-C2.

In certain mice in which the challenge tumor implant failed to grow following tumor ligation, it was observed that complete destruction of the first tumor had not been accomplished, as shown by ultimate progressive growth under the area where the tumor had been ligated. It thus appeared that ligation of a tumor mass induced resistance against reimplanted tumor tissue but that established tumor tissue was not influenced. Experiments were made to study the fate of tumor fragments of S215 implanted before, on the same day as, and at intervals following ligation of existing tumors. In mice in which reimplantation on the opposite flank was made 6 days before ligation of the tumor on the other flank (ten mice), or 2 days (twelve mice), or on the day of ligation (twelve mice), all the test implantations grew. In mice challenged 2 days (ten mice), or 6 days (ten mice) after ligation of the existing tumor none developed progressive tumors. Similar results were obtained with mice tested with S339; eight mice which received tumor implants 2 days before ligation of existing tumors

TABLE 1

IMMUNITY IN C3H-HE MICE FOLLOWING INDUCED REGRESSION OF SARCOMAS WHICH AROSE IN THIS STRAIN

Tumor	Transplant generation used for first graft	Tumor regressed following ligation	Transplant generation of tumor used for challenge	Mice in which tumor regressed immune upon reimplantation	Growth of tumor in untreated mice*
AC 304	1	6/7† (12)	2	0/6	6
AC 304-1	1	10/12 (14)	2	0/10	6
	2	9/9 (16)	3	0/9	6
AC 322	1	6/6 (14)	2	0/6	6
	2	6/6 (12)	2	0/6	6
S 215	2	8/8 (12)	2	7/8‡	6
	2§	9/9 (21)	5	2/9	6
	2§	6/6 (10)	5	3/6	6
	3	2/2 (10)	4	2/2	3
	4	8/9 (14)	5	5/8	6
	5	5/6 (6)	6	5/5	3
	7	6/6 (10)	9	6/6	5
S 339	2	12/15 (5)	2	11/12	6
S 299-B2	2	5/5 (19)	2	5/5	4
S 290-C1	1	4/7 (24)	1	4/4	4
S 290-C2	1	4/4 (6)	1	4/4	5
	2	6/8 (7)	2	6/6	4
S 303	2	6/6 (10)	2	5/6	6

* All controls died of progressive tumors.

† Numerator = Tumor regressed following ligation. Denominator = Number ligated.

‡ Numerator = No growth or regression of tumor. Denominator = Number of mice challenged.

§ The tumor used in these experiments was transplanted from very large, old tumors at the first and second transplant generation, and the mice were challenged with the same tumor which had been repeatedly transplanted at 10-day intervals.

Figures in parentheses represent days elapsed between beginning of ligation and challenge reimplantation of tumor.

developed progressive growths, while eight implanted 2 days after ligation failed to develop tumors.

Mice in which mammary tumors AC 304, AC 304-1, and AC 322 were destroyed by ligation developed progressive tumors when reimplanted 12–16 days after ligation.

DISCUSSION

The results of the above experiments confirm and extend those previously reported (2), showing that destruction of spontaneous mammary carcinomas of C3H-He mice by ligation does not induce immunity to progressive growth of the same tumor subsequently implanted. They further show that ligation of methylcholanthrene-induced sarcomas which arose in this strain of mice regularly induced a state of immunity which prevented growth of the same tumors when fragments were later implanted. This was observed with six unselected tumors which arose in mice treated with carcinogen, and immunity was induced against all these tumors at either the first or second transplant generation. Gross (3) and Lewis *et al.* (6, 7) have previously reported on the development of immunity in inbred mice and rats with methylcholanthrene-induced tumors. Those results have been explained as being due either to mutation of the tumor during repeated transplantation over long periods of time or to immunogenetic differences in the strains of animals used (4, 8). In the present work immunity was demonstrated before repeated transplants had been made, and, therefore, the chance of mutation during passage was minimized. The possibility of heterogeneity in the mice of the C3H-He stock cannot be completely ruled out, but, if the stock is heterogeneous, this factor does not influence the response of the mice to transplanted mammary tumors, for, in repeated trials with such tumors, no instance of immunity resulted after ligation, whereas immunity usually followed ligation of methylcholanthrene sarcomas.

Further study will be required to explain the different behavior of the two types of tumors, and the possibility that the methylcholanthrene-induced sarcomas represent a mutated tissue immunologically distinguishable from that of the strain of mouse in which they arose, and that a comparable mutation is not a characteristic of the spontaneous mammary tumors of C3H-He mice we have studied, should be considered. Such an event is consistent with the somatic mutation theory proposed by Strong (9), who has pointed out that carcinogen-induced tumors show evidence of mutation more rapidly than do spontaneous tumors (10). In this regard, attention should be called to the studies of Kirschbaum and Bittner (5), who described histological differences between methylcholanthrene-induced and spontaneous mammary tumors which arose in DBA mice.

Under the conditions of these experiments, the immunity induced by the ligated methylcholanthrene-induced sarcomas appears to be operative only in preventing progressive growth of tumor tissue implanted subsequent to ligation of the existing tumor; tumors implanted previously, and presumably established, were not adversely affected.

SUMMARY

Experiments are described in which it was shown that immunity of C3H-He mice resulted following ligation and atrophy of methylcholanthrene-induced sarcomas which arose in mice of this inbred strain. The results are discussed in relation to previously reported experiments in which C3H-He mice were not immunized against mammary carcinoma by similar methods.

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Attempts To Isolate the Active Agent in *Cysticercus fasciolaris**

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Published reports (1–4) have shown that sarcoma is an almost inevitable complication of infestation of the liver or subcutaneous tissues of rats with *Cysticercus fasciolaris*, the larva of the common tapeworm of the cat. A subsequent report (5) showed that the active agent was present in washed freshly ground parasites. Ninety per cent of the rats of the same inbred line as the host of the parasites died with multiple peritoneal sarcomas in an average of 89 days after intraperitoneal injection with a saline suspension of the freshly ground parasites obtained from benign cysts in hosts bearing an induced Cysticercus sarcoma. The present paper reports some attempts to isolate the active agent from similar suspensions of ground larvae.

MATERIALS AND METHODS

Living parasites were removed from benign cysts in the liver of rats with gross Cysticercus-induced sarcomas. The bearers of Cysticercus sarcomas may have from one to 100 or more parasites, but usually only one is primarily involved in the malignant process. Care was taken to select tumor-bearing hosts with few or no peritoneal metastases, and the parasites were removed only from the cysts that showed no gross involvement in the malignant process. Observations are also included (last table) on two groups of rats that were given injections of suspensions of larvae obtained from hosts in which no gross malignant transformation could be observed. In one group the age of the larvae—i.e., the duration of the infestation of the host—was 6–10 months. In the other it was 12 or more months. Primary Cysticercus tumors have been observed in a minimum of 8 months after infestation, but they are rare in rats that have been infested less than 10 months.

The larvae were washed in several successive volumes of sterile saline, then cut to a fine mash with scissors, and ground in a mortar before being suspended in the fluid medium to be used in the experiment. The suspending medium usually equaled 1 cc. to each two larvae, and approximately 1 cc. was injected intraperitoneally into each rat.

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In some instances aliquots of the suspension were subjected to temperature changes or were centrifuged at 2,000 r.p.m., and the decanted supernatant fluid was injected or filtered and then injected. In the latter cases the sediment was reconstituted to the original volume, and 1 cc. was injected into each of several rats. In other cases an aliquot of the original suspension remained at room temperature and was injected at the conclusion of the experimental procedure.

Except for one experiment, the injected rats were pedigreed young adult males and females of Fischer line 344. The eighteen injected rats of a foreign strain were rats of A × C line 9935, a totally unrelated strain. Data are also included for a group of Fischer line 344 rats that were given injections when 12–30 days of age. All the rats received the laboratory stock diet of Ralston Purina Laboratory Chow or Friskie Dog Pellets, supplemented with a green vegetable once a week and free access to water.

At necropsy a thorough post mortem examination included a description of all gross tumors and preservation of representative sections of each for confirmation by microscopic examination. Some of the rats had spontaneous neoplasms consistent with the expectation for untreated rats of this line. The frequency is indicated in Tables 1–4, and a key to their classification is given under Table 1. The negative findings were confirmed by microscopic examination of representative sections of omentum, mesentery, and diaphragm.

RESULTS

The results are summarized in Tables 1–4. The data presented in Table 1 show that 76 per cent of 105 rats given intraperitoneal injections of 1 cc. of a crude saline suspension of washed, freshly ground cysticerci died with multiple peritoneal sarcomas in an average of 133 days. Only 41 per cent of 86 rats injected with distilled water suspensions of similarly prepared larvae died with multiple peritoneal sarcomas, and the average latent period was 188 days.

Smaller numbers of rats were injected with similarly treated larvae suspended in 5 and 15 per cent sucrose, and phosphate buffer solutions[1] adjusted to pH 7.2 or 7.4. These suspensions appeared to be as effective as saline suspensions. Eighty-eight, 100, 66, and 56 per cent, respectively, of the injected rats died with multiple peritoneal sarcomas in an average of about 150 days. Ground larvae suspended in phosphate buffer solutions adjusted to pH 6.6, 6.8, and 7.0 appeared to be less effective.

[1] The phosphate buffer solutions were made according to Sorensen's phosphate standards of M/15 Na_2HPO_4 and M/15 KH_2PO_4. The solution adjusted to pH 7.0, being composed of 61.1 cc. of the former and 38.9 cc. of the latter.

Forty, 43, and 11 per cent, respectively, developed peritoneal sarcomas in an average latent period exceeding 200 days.

Of the 278 rats injected with these various crude suspensions of ground larvae, 163 or 59 per cent died with multiple peritoneal sarcomas in an average of 152 days. As a check for the possibility that the larvae might be contaminated with tumor cells from the primary host, 26 additional rats were given intraperitoneal injections of 1 cc. each of the sediment from the saline or water used as the final wash of the larvae. None of these rats developed peritoneal tumors.

The data presented in Table 2 represent attempts to test the stability of the active agent in saline, water, and phosphate buffer solutions. The controls, i.e., the crude suspensions that were injected immediately or after standing for 1 or 2 hours at room temperature, were also included in Table 1. Only 6 or 40 per cent of the fifteen rats

TABLE 1

THE INCIDENCES OF MULTIPLE PERITONEAL SARCOMAS IN RATS INJECTED WITH GROUND WASHED LARVAE SUSPENDED IN VARIOUS MEDIA

Suspending medium	No. rats	Per cent with sarcomas	Latent period Min.	Latent period Max. (days)	Latent period Mean	Mean survival negatives (days)	No. with spon. tumors
Saline	105	76	45	590	133	392	5 (a,c,f,m,n)
Distilled water	86	41	66	506	188	362	3 (c,i,l)
5 per cent sucrose	8	88	38	206	120	485	1 (d)
15 per cent sucrose	5	100	126	181	156		0
Buffer, pH 6.6	20	40	161	459	236	350	0
Buffer, pH 6.8	7	43	159	244	211	345	0
Buffer, pH 7.0	9	11	204	204	204	374	1 (b)
Buffer, pH 7.2	29	66	40	463	130	291	1 (k)
Buffer, pH 7.4	9	56	57	197	159	406	0
Sum	278	59	38	590	152	364	11
Final wash water	26	0				436	3 (b,e,j)

Key to the classification of spontaneous neoplasms in Tables 1–4.

a = Lymphosarcoma of mesenteric lymph notes.
b = Fibroadenoma of mammary gland.
c = Leukemia.
d = Adenocarcinoma of adrenal.
e = Sarcoma of leg.
f = Sarcoma of testicle.
g = Subcutaneous fibroma.
h = Adenoacanthoma of mammary gland.
i = Adenocarcinoma of lung.
j = Angiosarcoma of uterus.
k = Lymphosarcoma of neck.
l = Papillary carcinoma of bladder.
m = Sarcoma of lung.
n = Squamous-cell carcinoma of the orbit.

TABLE 2

THE INCIDENCES OF MULTIPLE PERITONEAL SARCOMAS IN RATS INJECTED WITH GROUND WASHED LARVAE SUSPENDED IN VARIOUS MEDIA AND INCUBATED AT SEVERAL TEMPERATURES

Group	No. rats	Per cent with sarcomas	Latent period Min.	Latent period Max. (days)	Latent period Mean	Mean survival negatives (days)	No. with spon. tumors
Saline, immediately	15*	40	112	590	333	428	0
" 1–2 hrs. R.T.†	5*	100	72	217	140		1 (m)
" 1 hr. 38° C.	5	0				432	0
" 48 hrs. −70° C.	7	0				431	0
" 6 days −70° C.	9	0				536	0
Water, immediately	13*	38	77	239	136	245	1 (e)
" 2 hrs. R.T.	12*	25	121	199	147	339	0
" 1 hr. 58° C.	8	0				289	1 (a)
" −70° C.	3	100	50	59	53		0
" 1 hr. −70° C.	14	29	52	63	58	200	0
" 2 hrs. −70° C.	15	0				321	1 (c)
" 24 hrs. −70° C.	10	0				368	4 (a,c,f,g)
Buffer pH 6.6–7.0, immediately	19*	47	161	459	241	370	1 (b)
2 hrs. R.T.	17*	18	159	205	185	348	0
Buffer pH 7.2–7.4, immediately	15*	73	40	197	114	241	0
2 hrs. R.T.	15*	73	51	210	105	336	0
Sum	182	33	40	590	156	349	9

* Included also in Table 1.
† R.T. = room temperature.

that were injected immediately with a saline suspension of fresh larvae died with peritoneal sarcomas, and the average latent period was 333 days. This percentage of rats with peritoneal sarcomas was considerably below the expectation from previous experiments with saline suspensions, and the latent period was more than doubled. The previous observations were based on injections made about 2 hours after the larvae were removed from the liver. The time lapse had been considered a necessary hazard to the activity, but in this experiment the activity appeared to increase after standing at room temperature. The same suspension after standing for 2 hours at room temperature produced sarcomas in all five injected rats in an average of 140 days. After 1 hour in the incubator at 38° C. the suspension was completely inactive. Larvae from the same source that were frozen at −70° C. and maintained at that temperature made inactive suspensions after 48 hours and 6 days.

When the ground larvae were suspended in water and injected immediately, five of thirteen rats or 38 per cent died with multiple peritoneal sarcomas in an average of 136 days. Similar results were obtained with the same suspension after it had stood for 2 hours at room temperature. That is, three of twelve rats or 25 per cent died with peritoneal sarcomas in an average of 147 days, indicating that, if there were any change as a result of standing at room temperature, it was detrimental to the activity. When this suspension was heated to 58° C. for 1 hour it was completely inactive.

Larvae that were frozen at −70° C., ground immediately and suspended in water, produced peri-

TABLE 3

THE INCIDENCES OF MULTIPLE PERITONEAL SARCOMAS IN RATS INJECTED WITH VARIOUS FRACTIONS OF GROUND WASHED LARVAE SUSPENDED IN SEVERAL MEDIA

Group	No. rats	Per cent with sarcomas	Latent period Min. (days)	Latent period Max. (days)	Latent period Mean	Mean survival negatives (days)	No. with spon. tumors
Water, crude	6*	67	83	110	95	470	0
" supernatant	33	18	77	499	168	472	1 (b)
" recon. sediment	31	67	50	520	150	423	0
" filtered supernat.	23	0				492	2 (a,b)
5 per cent sucrose, crude	8*	88	38	206	120	485	1 (d)
" supernatant	14	0				373	1 (a)
" recon. sediment	12	83	38	416	138	294	0
15 per cent sucrose, crude	5*	100	126	181	156		0
" supernatant	5	0				407	0
" recon. sediment	5	20	150	150	150	260	0
Buffer pH 7.2, crude	29*	66	40	463	130	291	1 (k)
" supernatant	6	0				394	0
" recon. sediment	6	67	49	390	138	132	0
" filtered supernat.	7	0				469	0
Homologous serum, crude	3	67	60	144	102	710	0
" supernatant	6	0				510	0
" recon. sediment	5	0				225	0
Water, tumor cell extract							
" supernatant	4	0				152	0
" Berkfeld V filtrate	6	0				204	0
" Berkfeld W filtrate	4	0				309	0

* Included also in Table 1.

TABLE 4

THE INCIDENCES OF MULTIPLE PERITONEAL SARCOMAS IN RATS OF A FOREIGN STRAIN, YOUNG AND OLDER RATS OF THE SAME STRAIN, AND IN RATS INJECTED WITH BENIGN LARVAE

Group	Fluid medium	No. rats	Per cent with sarcomas	Latent period Min. (days)	Latent period Max. (days)	Latent period Mean	Mean survival negatives (days)	No. with spon. tumors
Foreign strain A×C line 9935	Saline	18	0				369	1 (f)
Same strain 12–30 days	Water	72	29	75	669	268	467	3 (a,b,h)
Same strain 3–12 months	Water	41*	51	66	506	209	360	0
Benign larvae 6–10 months	Water	63	0				336	3 (d,i,l)
Benign larvae 12 mo. or more	Water	30	33	87	210	167	313	1 (a)

* Included also in Table 1.

toneal sarcomas in all three injected rats in an average of only 53 days. Larvae ground after 1 hour at −70° C. and suspended in water produced sarcomas in four of fourteen injected rats in an average of 58 days. Frozen larvae maintained at −70° C. for 2 or more hours were completely inactivated.

Suspensions made with phosphate buffer solutions adjusted to pH 6.6–7.0 were more effective immediately than after 2 hours at room temperature. Nine of nineteen rats injected immediately died with peritoneal sarcomas in an average of 241 days, while only three of seventeen developed tumors when injected with the suspension after it had remained at room temperature for 2 hours. Larval suspensions made with phosphate buffer solutions adjusted to pH 7.2–7.4 were equally effective immediately or after 2 hours at room temperature. In each case, eleven or 73 per cent of fifteen injected rats died with multiple peritoneal sarcomas in an average of 114 and 105 days, respectively.

Experiments previously reported (5) indicated that the active agent might be eluted from the sediment of the saline suspension of larvae after it had been centrifuged for 10 or more minutes at 2,000 r.p.m. The decanted supernatant fluid showed some activity, but the reconstituted sediment remained as active as the original suspension. No activity was observed in the filtrates of the supernatant fluid, but considerable difficulty was experienced in passing the fluid through Berkfeld V or W filters. The data presented in Table 3 represent some attempts to find a more suitable medium for eluting the active agent. Distilled water, however, proved to be the only eluant tested in the current experiments with which an active supernatant fluid was obtained. In this experiment four of six rats given injections of an aliquot of the original aqueous suspension at the conclusion of the experiment died with multiple peritoneal sarcomas in an average of 95 days. Only six of 33 rats that received the decanted supernatant fluid after the suspension had been centrifuged for 10 minutes at 2,000 r.p.m. developed peritoneal sarcomas. The reconstituted sediment showed the same activity as the original suspension—i.e., 21 or 67 per cent of the 31 injected rats died with peritoneal sarcomas in an average of 150 days. None of the 23 rats that were injected with the supernatant fluid after it had passed through Seitz clarifying and sterilizing filter pads developed peritoneal sarcomas.

The fluids decanted from suspensions of larvae in 5 and 15 per cent sucrose, phosphate buffer adjusted to pH 7.2, and homologous serum after centrifugation for 10 minutes at 2,000 r.p.m. showed no activity. The sediment reconstituted in 5 per cent sucrose and pH 7.2 phosphate buffer showed the same activity as the original suspensions, while that which was reconstituted in 15 per cent sucrose showed decreased activity and that which was reconstituted in homologous serum showed no activity.

Only negative results were obtained when an aqueous suspension of tumor cells from one of the induced peritoneal sarcomas was centrifuged for 10 minutes at 2,000 r.p.m. and the decanted supernatant fluid was injected intraperitoneally either before or after passing it through Berkfeld V and W filters.

The previous report (5) showed that unrelated rats responded less quickly and less frequently to aqueous or saline suspensions of ground larvae than rats of the same inbred line as the host of the parasites. Table 4 shows that eighteen rats of A × C line 9935, a totally unrelated strain, were completely negative, although they survived an average of 369 days after injection. Rats of the same inbred line that were under 30 days of age when injected were no more susceptible than older rats of the same strain. Of 72 rats injected with an aqueous suspension of larvae when they were 12–30 days of age, 29 per cent died with peritoneal sarcomas, while 51 per cent of 41 rats that were simultaneously injected when they were 3 months to 1 year of age died with peritoneal sarcomas. The minimum latent period was 75 days for the young rats and 66 days for their older sibs.

Aqueous larval suspensions made with parasites that were 6–10 months of age when they were removed from rats that had not developed primary Cysticercus tumors did not initiate any peritoneal sarcomas in 63 injected rats. Aqueous suspensions made with larvae that were 12 or more months of age when they were removed from rats in which no primary Cysticercus tumor was observed produced peritoneal sarcomas in ten of 30 injected rats. The average latent period was 167 days; the minimum latent period of 87 days was not greatly different from the average obtained for aqueous suspensions made from larvae from hosts that had gross Cysticercus sarcomas.

DISCUSSION

As in the previous experiments (5), the currently reported results indicate that a highly labile agent capable of inducing sarcoma when introduced into the peritoneal cavity of rats may be present in larvae of *Taenia taeniaeformis* that have resided 12 or more months in the liver of other rats of the same strain. Washed, freshly ground larvae suspended in saline, sucrose solutions, and phosphate buffer solutions adjusted to pH 7.2 and 7.4

were about equally effective whether they were injected immediately or after standing 2 hours at room temperature. Ground larvae suspended in distilled water or phosphate buffer solutions adjusted to pH 6.6–7.0 showed less activity after 2 hours at room temperature than when they were injected immediately. Incubation of the suspension for 1 hour at 37° C., heating it to 58° C., or storing the larvae at −70° C. for 2 or more hours destroyed the activity.

The supernatant fluid that was decanted after the suspension of ground larvae had been centrifuged for 10 minutes at 2,000 r.p.m. was, with few exceptions, inactive. It was inactive in all instances after being passed through Seitz sterilizing filter pads.

The reconstituted sediment, consisting chiefly of the calcium carbonate corpuscles characteristically found in the coelom of the parasites, was with few exceptions as effective as the original suspension. These corpuscles as shown in Figures 1 and 2 can usually be identified in the peritoneal tissues of the rat even many months after injection. In rats without tumors they are usually surrounded by a fibrous capsule, and the induced sarcomas appear to arise in close proximity to them, as shown in Figures 3 and 4. If the active agent is adsorbed to the surface of these corpuscles, the current attempts to elute it have been mostly unsuccessful. The corpuscles, on the other hand, may serve as the physical means of keeping the active agent in contact with the peritoneal tissues for the 5 or more months that, on the average, precede the development of the tumors. In the absence of the corpuscles the supernatant fluid and the filtered solutions might fail to elicit tumor formation because of the too rapid absorption and elimination of the agent from the peritoneal tissues.

SUMMARY

1. Multiple peritoneal sarcomas were observed in 163 or 59 per cent of the rats injected intraperitoneally with 1 cc. of an aqueous, saline, sucrose, or phosphate buffer suspension of washed, freshly ground taenia larvae obtained from rats of the same strain bearing an induced Cysticercus sarcoma. The average latent period was 152 days.

2. At room temperature saline, sucrose, and phosphate buffer solutions adjusted to pH 7.2 and 7.4 made more stable suspensions than distilled water or phosphate buffer solutions adjusted to pH 6.6 to 7.0.

3. Incubation of a saline suspension for 1 hour at 37° C., heating an aqueous suspension to 58° C., or storing the larvae at −70° C. for 2 or more hours destroyed all activity.

4. The decanted supernatant fluid from an aqueous suspension of larvae centrifuged for 10 minutes at 2,000 r.p.m. induced peritoneal sarcomas, but no sarcomas resulted from the injection of supernatant fluid from similarly treated suspensions made with sucrose, phosphate buffer, or homologous serum. In most experiments the reconstituted sediment was as active as the original suspension. No tumors resulted from the injection of any supernatant fluid after it had passed through Seitz clarifying and sterilizing filter pads.

5. Rats of a foreign strain did not develop tumors, and rats of the same strain as the host of the parasites that were injected when they were 12–30 days of age were no more susceptible than their older sibs.

6. Larvae 6–10 months of age when removed from hosts with no visible primary tumor made inactive aqueous suspensions, but similar larvae 12 or more months of age made as active aqueous suspensions as larvae from hosts with gross Cysticercus sarcomas.

7. No peritoneal tumors were induced by the intraperitoneal injection of the sediment from the fluid used for the final washing of the parasites or from the injection of the decanted supernatant fluid from an aqueous suspension of induced peritoneal sarcoma cells before or after passage through Berkfeld V or W filters.

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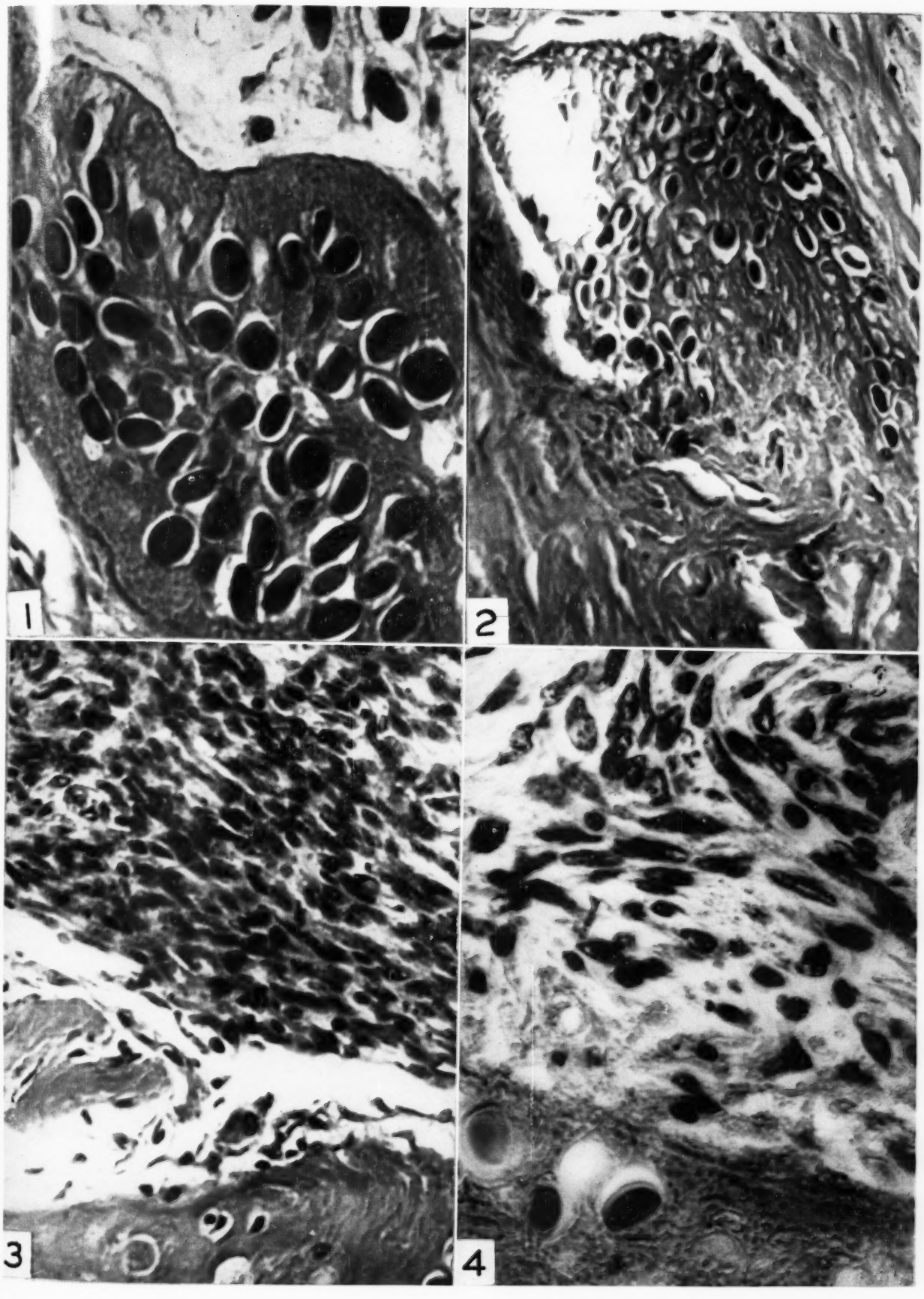
Fig. 1.—Calcium carbonate corpuscles from the mesentery of a rat 423 days after injection of a saline suspension that was made from larvae that had been frozen at −70° C. for 24 hours. × 300.

Fig. 2.—Fibrous reaction around corpuscles from the same rat as shown in Figure 1. × 150.

Fig. 3.—Spindle-cell sarcoma around corpuscles 107 days after the injection of a saline suspension of fresh, ground larvae. × 150.

Fig. 4.—Spindle-cell sarcoma around injected material 101 days after the injection of an aqueous suspension of freshly ground larvae. × 300.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Estimation of the Antileukemic Potency of the Antimetabolite Aminopterin, Administered Alone and in Combination with Citrovorum Factor or Folic Acid*

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Administration of citrovorum factor (CF) or pteroylglutamic acid (FA) may reduce the lethal toxicity for mice of the antimetabolite 4-aminopteroylglutamic acid (aminopterin). The mortality response is dependent upon the relative dose level of FA or CF administered with the aminopterin and is influenced by the temporal relationships of administration of the drugs (1, 8–12).

Administration of FA or CF may also interfere with the tumor inhibitory effects of 4-amino FA derivatives (2, 3, 5, 13, 14). However, the tumor studies were confined to the effect of FA and CF on the therapeutic action of a dose of antagonist in the range of the maximum tolerated dose on multiple treatment. Consideration was not given to the relationship of host toxicity to antitumor activity of the drugs.

Since toxicity of aminopterin to the host has constituted a limiting factor in the treatment of neoplasia, studies were undertaken to determine in what manner the quantitative host-toxicity relationships demonstrated with FA, CF, and aminopterin may relate to their effects on the growth of tumor. In this investigation a comparison is made of the relative effectiveness of the following treatments in prolonging the survival time of mice with a transplantable leukemia.

a) Aminopterin alone.

b) Aminopterin plus concomitantly administered CF.

c) Aminopterin plus FA, when the FA is administered 1 hour prior to the aminopterin.

d) Aminopterin plus concomitantly administered FA.[1]

For this purpose, an experimental design was employed and an analysis developed which permit comparisons of drug effect on survival time of animals that succumb to the tumor for equivalent host toxicity resulting from drug treatment.

METHODS

General.—The experiments were conducted with DBA male mice, subline 212, inoculated with the lymphoid leukemia L 1210 (6, 7, 14). The animals were given inoculations aseptically in the right thigh of 0.1 ml. of a uniform saline suspension of tumor cells. Aminopterin and FA were administered in 2 per cent and CF in 0.5 per cent sodium bicarbonate. For a single injection, the total volume in ml. of solution for each drug employed corresponded to 1 per cent of the body weight of the mouse. The drugs were administered subcutaneously in opposite axillae. In each experiment the system of randomization previously described, in which both the animals and order of injection of the drugs were randomized, was employed (9). This was done on the day of drug treatment. The animals were maintained at laboratory temperature (72°–78° F.), in plastic cages, were offered Purina chow and water ad libitum, and were observed daily for mortality.

Experimental.—The dosages and the temporal relationships of administration of the drugs are included with the detailed data for the individual experiments. The dosage of aminopterin, with or without CF or FA, was intended to cover, as effectively as possible, the 0–100 per cent range for toxic mortality.[2]

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[1] Aminopterin, folic acid, and citrovorum factor (Leucovorin; calcium salt containing 5 molecules of water of hydration; designated in this paper as CF) were kindly provided by the Lederle Laboratories Division of the American Cyanamid Co.

[2] In some cases the 0–100 per cent toxic mortality range was not entirely covered. This may have occurred because of differences in susceptibilities of mice in different experiments, possible differences in the conditions of experimentation or in

The experiments were designed to provide a temporal separation of deaths resulting from drug toxicity and deaths resulting from tumor growth. This was accomplished in the following manner. The drugs were administered 3 days following tumor implantation. Most aminopterin toxicity deaths, with or without administered CF, occur from 3 to 6 days following administration of the drugs (9) (6–9 days following tumor inoculation). Only occasional deaths from drug toxicity occur as late as 10 days after administration of aminopterin (13 days following tumor inoculation). The concentration of tumor inoculum was such as to insure virtually 100 per cent takes, with deaths from the tumor in control mice not beginning to occur until the 14th–16th day following tumor inoculation. The difference between the time of death from drug toxicity and the time of death from the tumor permits almost complete separation of the two categories of mortality. Occasional borderline cases could be separated on the basis of tumor size at the site of inoculation and the weight history of the mouse prior to death.[3] In the current experiments only those deaths which occurred prior to the earliest control tumor deaths in the particular experiment were classified as drug toxicity deaths.

Statistical.—The employment of a series of dose levels of aminopterin and the separation of drug toxicity deaths from tumor deaths provides a basis for answering the following question: Of two treatments giving the same percentage toxic mortality, which is more effective in prolonging the average survival time of animals that succumb to the growth of the tumor?

The first step in the statistical analysis is the calculation of the probit of toxic mortality for each dose of aminopterin, administered alone or in combination with CF or FA. In experiments involving aminopterin alone, together with aminopterin plus a series of dose levels of CF, the calculated probits were obtained primarily by fitting a maximum likelihood modified probit plane of the form, $Y = a + b \log (\text{aminopterin}/[C + CF])$[4] to the toxicity data (4, 9). When aminopterin alone, together with aminopterin plus a single dose level of CF or FA, was employed, fitting of a probit plane was reduced simply to fitting a common probit slope for the toxicity data of the classes of treatment employed.

The second step in the statistical analysis is the fitting of a linear relationship between average time to death from tumor for mice surviving aminopterin toxicity and the probit of toxic mortality. For this purpose, least squares common slope lines were fitted relating the day of tumor death for each toxicity survivor to the calculated probit of toxic mortality, for the treatments received by the mouse.[5] Analysis of covariance procedures were employed to make statistical tests of the differences in the levels of the fitted lines.

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the potency of the aminopterin, and because of a change in the kind of CF employed as compared to earlier experiments (9).

[3] For this purpose the animals were weighed periodically, and the local tumor volume in the thigh was estimated by caliper measurements in three dimensions. These data are not reported here.

[4] Y = probit of per cent mortality. Aminopterin and CF expressed in moles/kg. C = endogenous reserve of protection, expressed as moles/kg CF administered simultaneously with aminopterin (9).

[5] In fitting these lines, the results for six treated mice could not be taken into account. These mice survived the experiment and were apparently tumor-free for the period of observation (3 months).

RESULTS

Experimental.—The results of four experiments are summarized in Tables 1–4 and in the corresponding Charts 1–4. In the first three experiments (Tables 1–3, Charts 1–3), CF was administered concomitantly with aminopterin. In the fourth experiment (Table 4, Chart 4), FA was administered 1 hour prior to aminopterin in one group and concomitantly with aminopterin in a second group.

The number of deaths from drug toxicity, the calculated probit of toxic mortality, and the day of death from tumor for mice surviving drug toxicity are listed for each dose of aminopterin, administered alone or with metabolite (Tables 1–4). Concomitant administration of CF (Tables 1–3) and prior administration of FA (Table 4) reduced the toxicity of aminopterin to the host, as previously reported (8, 9). These metabolite treatments resulted in a decrease in the probit of toxic mortality for a constant dose level of aminopterin (Tables 1–4). Concomitant administration of FA was ineffective (Table 4) (8).

The least squares common slope lines relating time to death from tumor of toxicity survivors to the probit of toxic mortality are shown for each of the four experiments (Charts 1–4). Also shown on each chart is the over-all average time to death from tumor for all the control animals in the experiment.

For equivalent probit of toxic mortality, aminopterin was more effective than aminopterin plus concomitantly administered CF (Tables 1–3; Charts 1–3) in prolonging the survival time of animals which succumbed to the leukemia. This occurred not only when the mice treated with aminopterin received no additional treatment (Tables 1 and 2, Charts 1 and 2) but also when they were treated with CF vehicle (0.5 per cent sodium bicarbonate) (Table 3, Chart 3). In like manner, for equivalent probit of toxic mortality, aminopterin was more effective than FA plus aminopterin, when the FA was administered 1 hour early with respect to the aminopterin (Table 4, Chart 4). However, aminopterin was not more effective than FA plus aminopterin, when the FA and aminopterin were administered concomitantly (Table 4, Chart 4).

Statistical.—Though the true relationship between day of death from tumor and calculated probit of toxic mortality is undoubtedly not strictly linear, the fitting of linear relationships may nevertheless be usefully employed to evaluate differences resulting from treatment. The validity of the method is indicated by the failure to observe any significant indication of departure from linear-

TABLE 1

RESPONSE OF LEUKEMIC (L 1210) MICE TO CONCOMITANT ADMINISTRATION OF CITROVORUM FACTOR AND AMINOPTERIN* (EXPERIMENT 1)

(Treatment 3 days after tumor inoculation)

Dose (mg/kg)		Deaths from drug toxicity	Calculated probit of toxic	No. mice surviving drug toxicity and dying from tumor — Days after tumor inoculation												
CF	Aminopterin	No. mice treated	mortality†	15	16	17	18	19	20	21	22	23	24	25	26	.. 29
0	2.7	1/10	3.14				1	4	1		1	1	1			
0	4.0	2/10	3.90				1	4		1	1	1				
0	6.0	3/10	4.66						1	1	2	2				‡
0	9.0	7/10	5.42							1		1	1			
0	13.5	10/10	6.18							(no survivors)						
0	20.2	10/10	6.94							(no survivors)						
0	30.4	9/10	7.70										1			
3.75	9.0	1/10	4.19			3	2	3								1
3.75	13.5	2/10	4.95			5	3									
3.75	20.2	6/10	5.70					2	1		1					
3.75	30.4	9/10	6.46									1				
3.75	45.6	10/10	7.22							(no survivors)						
3.75	68.4	10/10	7.98							(no survivors)						
3.75	102	10/10	8.74							(no survivors)						
7.5	13.5	2/10	4.21			2	3	2	1							
7.5	20.2	4/10	4.97			1	2	1	1		1					
7.5	30.4	9/10	5.73					1								
7.5	45.6	10/10	6.49							(no survivors)						
7.5	68.4	10/10	7.24							(no survivors)						
7.5	102	10/10	8.00							(no survivors)						
7.5	154	10/10	8.76							(no survivors)						
15	30.4	4/10	4.03			2	1	1	1		1					
15	45.6	9/10	4.79							1						
15	68.4	9/10	5.55									1				
15	102	10/10	6.31							(no survivors)						
15	154	10/10	7.06							(no survivors)						
15	231	10/10	7.82							(no survivors)						
15	346	10/10	8.58							(no survivors)						
Controls																
7.5 mg/kg CF		0/10		3	2	5										
15 mg/kg CF		0/10			3	4	2	1								
2 per cent $NaHCO_3$		0/10			3	7										
Untreated		0/7			3	2	1		1							

* Inoculum—21,400 cells per mouse.

† Calculated probits obtained from the fitted maximum likelihood dose response modified probit plane (9). The modified probit plane was fitted using two cycles of an unpublished procedure developed by one of the authors, N.M.

‡ One mouse apparently tumor-free at 90 days.

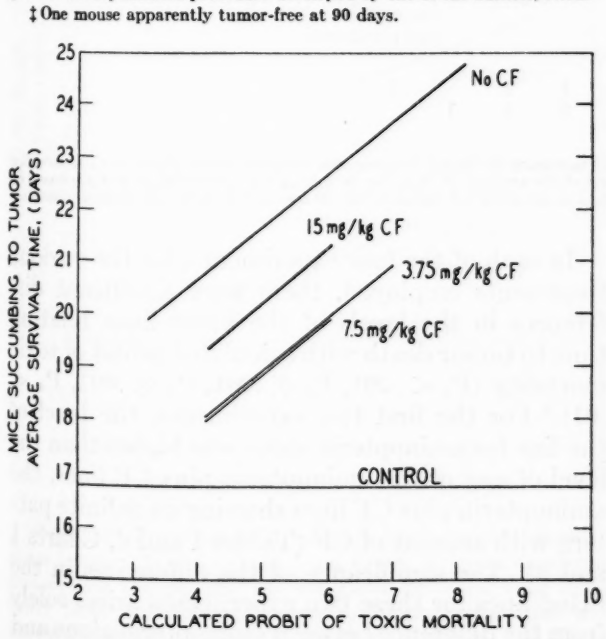

CHART 1 (Experiment 1).—Fitted least squares relationships between time to death from tumor for mice surviving aminopterin toxicity and the calculated probit of toxic mortality from aminopterin. Separate common slope lines are shown for varying amounts of concomitant CF. Lines are drawn to extend approximately over the range of calculated probits at which there were any survivors. Control line is based on the average tumor survival time for 34 mice receiving no aminopterin.

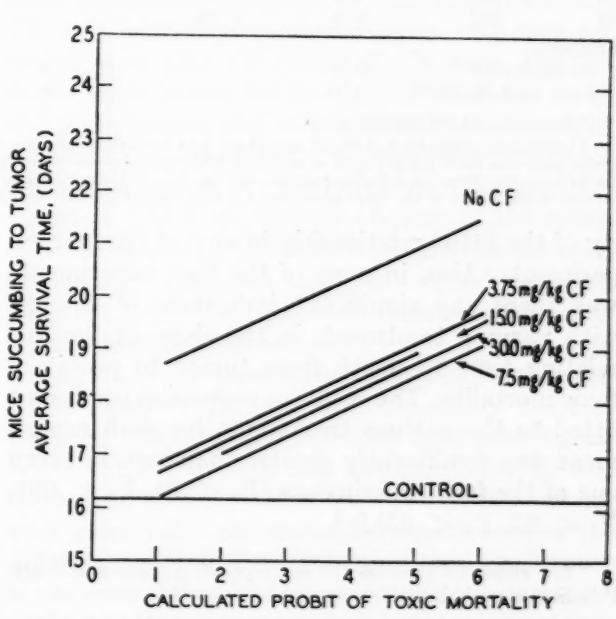

CHART 2 (Experiment 2).—Fitted least squares relationships between time to death from tumor for mice surviving aminopterin toxicity and the calculated probit of toxic mortality from aminopterin. Separate common slope lines are shown for varying amounts of concomitant CF. Lines are drawn to extend approximately over the range of calculated probits at which there were any survivors. Control line is based on the average tumor survival time for 22 mice receiving no aminopterin.

TABLE 2

RESPONSE OF LEUKEMIC (L 1210) MICE TO CONCOMITANT ADMINISTRATION OF CITROVORUM FACTOR AND AMINOPTERIN* (EXPERIMENT 2)

(Treatment 3 days after tumor inoculation)

Dose CF	Dose Aminopterin (mg/kg)	Deaths from drug toxicity No. mice treated	Calculated probit of toxic mortality†	No. mice surviving drug toxicity and dying from tumor Days after tumor inoculation 14	15	16	17	18	19	20	21	22	23	24	25	26	27
0	1.8	0/9	0.64				2	3	2	1	1						
0	2.7	0/9	1.56				3	1	2	1	1	1					
0	4.0	1/9	2.49				1	2	3	1					1		
0	6.0	2/9	3.41					1	2	1	1	1	1				
0	9.0	1/9	4.34					1	2	2	1	1	1				
0	13.5	5/9	5.26				1				1	1				1	
3.75	4.0	0/9	1.21		2	1	2	1	2	1							
3.75	6.0	0/9	2.13			2	5	1				1					
3.75	9.0	0/9	3.06		1	2	1	3	2								
3.75	13.5	0/9	3.98		1		1	2	4								1
3.75	20.2	3/9	4.91			1	1	3					1				
3.75	30.4	6/9	5.83				1			1	1						
3.75	45.6	9/9	6.76							(no survivors)							
7.5	6.0	0/9	1.32		2	2	3	1	1								
7.5	9.0	0/9	2.24			3	3	2	1								
7.5	13.5	0/9	3.17			3	3	1	1	1							
7.5	20.2	1/9	4.09			2	4	1	1								
7.5	30.4	4/9	5.02			1		1	1		1	1					
7.5	45.6	8/9	5.94							1							
15	9.0	0/9	1.17	1	1	3	3	1									
15	13.5	0/9	2.10			2	4	1	2								
15	20.2	0/9	3.02			1	2	3	1	1		1					
15	30.4	2/9	3.95			2	1	2	2								
15	45.6	4/9	4.87					1	1	2	1						
15	68.4	9/9	5.80							(no survivors)							
15	102	9/9	6.72							(no survivors)							
30	20.2	0/9	2.83			2	3	3		1							
30	30.4	1/9	3.75		1	1	2	1	1	2							
30	45.6	1/9	4.68				1		5	1		1					
30	68.4	8/9	5.60						1								
30	102	9/9	6.53							(no survivors)							
30	154	9/9	7.45							(no survivors)							
30	231	9/9	8.38							(no survivors)							
Controls																	
30 mg/kg CF		0/11		2	3	3	1	1		1							
2 per cent $NaHCO_3$		0/11		1	3	2	3	1	1								

* Inoculum—84,500 cells per mouse.

† Calculated probits for 0, 3.75, 7.5, and 15 mg/kg CF obtained from the fitted maximum likelihood dose response modified probit plane (9). The modified probit plane was fitted using 1 cycle of an unpublished procedure developed by one of the authors (N.M.). For 30 mg/kg CF calculated probits are based on the LD_{50} for the 30 mg/kg CF data coupled with the slope of the probit plane fitted to the other CF dosages. The 30 mg/kg CF data was treated separately because of the failure of this large amount of CF to go completely into solution.

ity of the fitted relationship in any of the four experiments. Also, in none of the four experiments was there any significant indication of change, with varying treatment, in the slope of the line relating time to death from tumor to probit of toxic mortality. The common regression coefficient fitted to the various treatments for each experiment was significantly greater than zero in every one of the four experiments ($P_1 < .01$, $P_2 \ll .001$, $P_3 < .05$, $P_4 < .05$).[6, 7]

In each of the four experiments, for the various treatments employed, there were significant differences in the levels of the fitted lines relating time to tumor death with calculated probit of toxic mortality ($P_1 < .001$, $P_2 \ll .001$, $P_3 \ll .001$, $P_4 < .01$).[6] For the first two experiments, the level of the line for aminopterin alone was higher than the level of any of the aminopterin plus CF lines, the aminopterin plus CF lines showing no definite pattern with amount of CF (Tables 1 and 2, Charts 1 and 2). The significance of the differences in the fitted lines for these two experiments arises solely from the difference between aminopterin alone and

[6] The subscript denotes the corresponding table and chart. P indicates probability.

[7] It is recognized that an artificial increase in average time to death from tumor could occur with increasing dose of aminopterin through a selective process in which drug toxicity effects are greatest for mice which would otherwise have died early from tumor. That no such selective process occurred is indicated by the observation that a large proportion of the tumor deaths for mice receiving aminopterin occurred beyond the range in time of tumor deaths for control mice not receiving aminopterin.

TABLE 3

RESPONSE OF LEUKEMIC (L 1210) MICE TO CONCOMITANT ADMINISTRATION OF CITROVORUM FACTOR AND AMINOPTERIN* (EXPERIMENT 3)

(Treatment 3 days after tumor inoculation)

Dose CF (mg/kg)	Dose Aminopterin (mg/kg)	Deaths from drug toxicity: No. mice treated	Calculated probit of toxic mortality†	No. of mice surviving drug toxicity and dying from tumor: Days after tumor inoculation 16	17	18	19	20	21	22	23	24	25	..	28	29	30	31
0‡	1.8	0/13	2.03			3	1	3	3		1	1	1					
0	2.7	2/13	3.09			2		3	2	2					1			1
0	4.0	3/13	4.14			1	1	1	3	1	1					1		1
0	6.0	5/13	5.19				1		1	3	1	2						
0	9.0	11/13	6.24					1				1						
0	13.5	13/13	7.29							(no survivors)								
0	20.2	13/13	8.35							(no survivors)								
15	9.0	0/13	1.81		6	4	1	2										
15	13.5	0/13	2.86		7	1	5											
15	20.2	0/13	3.91		2	5	4	2										
15	30.4	6/13	4.96			1	3	2	1									
15	45.6	13/13	6.02							(no survivors)								
15	68.4	13/13	7.07							(no survivors)								
15	102	13/13	8.12							(no survivors)								
Controls																		
2 per cent $NaHCO_3$		0/13		8	3	1		1										
0.5 per cent + 2 per cent $NaHCO_3$		0/12		5	4	2		1										
Untreated		0/12		4	4	3							1					

* Inoculum—105,000 cells per mouse.

† Calculated probits obtained from the fitted maximum likelihood dose response curve. A 2-way common probit slope curve was fitted using 2 cycles of the Cornfield-Mantel procedure (4).

‡ 0 CF indicates treatment with vehicle only, 0.5 per cent $NaHCO_3$.

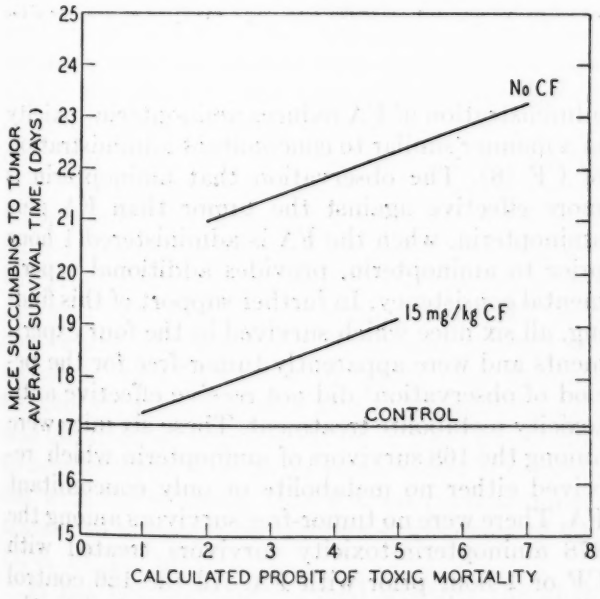

CHART 3 (Experiment 3).—Fitted least squares relationships between time to death from tumor for mice surviving aminopterin toxicity and the calculated probit of toxic mortality from aminopterin. Separate common slope lines are shown for 0 CF and for 15 mg/kg concomitant CF. Lines are drawn to extend approximately over the range of calculated probits at which there were any survivors. Control line is based on the average tumor survival time for 37 mice receiving no aminopterin.

aminopterin plus CF, with no significant differences existing among the groups receiving aminopterin plus the varying levels of CF. For the third experiment, the line for aminopterin without CF was significantly higher than the line for aminopterin plus CF (Table 3, Chart 3). For the fourth experiment, the lines for aminopterin alone and aminopterin plus concomitantly administered FA were close, both being higher than the line for aminopterin plus FA administered 1 hour earlier (Table 4, Chart 4). The significance of the difference, here, arose solely from the difference between noneffective (zero FA and concomitant FA) and effective treatment (prior administration of FA) against aminopterin toxicity.

The statistical significance tests employed in the above analysis did not take account of the fact that the toxic mortality probits used were themselves subject to sampling variation. Taking this factor into account would tend to weaken, but not alter essentially, the statistical significance of the differences noted. Strengthening this significance is the consistency of the observation in three experiments that, for equivalent host toxicity, treatment with aminopterin alone is more efficacious against the tumor than treatment with aminopterin plus concomitantly administered CF. Prior

TABLE 4

RESPONSE OF LEUKEMIC (L 1210) MICE TO CONCOMITANT AND TO PRIOR ADMINISTRATION OF FOLIC ACID WITH AMINOPTERIN* (EXPERIMENT 4)

(Treatment 3 days after tumor inoculation)

Concomitant FA	Dose 1 hour prior FA (mg/kg)	Aminopterin	Deaths from drug toxicity No. mice treated	Calculated probit of toxic mortality†	No. mice surviving drug toxicity and dying from tumor — Days after tumor inoculation 14	15	16	17	18	19	20	21	22	23	24	25	26
0	0	1.8	7/13	4.37				2	1		1		1	1			
0	0	2.7	5/13	4.87					2	1	2		1		1		1
0	0	4.0	10/13	5.37						1			2				
0	0	6.0	7/13	5.86						1				1	1		1‡
0	0	9.0	12/13	6.36		1											
0	0	13.5	13/13	6.86						(no survivors)							
0	0	20.2	12/13	7.35											1		
25	0	1.8	6/13	4.36				1	1	1	1		2	1			
25	0	2.7	7/13	4.86				1		3				1			
25	0	4.0	5/13	5.36				1			3	1	1	1			§§§
25	0	6.0	9/13	5.86								1		2			
25	0	9.0	12/13	6.35								1					
25	0	13.5	13/13	6.85						(no survivors)							
25	0	20.2	13/13	7.35						(no survivors)							
25	0	30.4	13/13	7.84						(no survivors)							
0	25	6.0	0/13	3.46			1	4	3	3	2						
0	25	9.0	(Excluded because of extraneous damage to mice)														
0	25	13.5	2/13	4.45				2	4	4	1						
0	25	20.2	2/13	4.95			1	4	4		1				1		
0	25	30.4	12/13	5.44							1						
0	25	45.6	13/13	5.94						(no survivors)							
0	25	68.4	13/13	6.44						(no survivors)							
Controls																	
25 mg/kg FA			0/10			1	5	3				1					
2 per cent $NaHCO_3$			0/10				5	2	2	1							
Untreated			0/10		1		2	3	2		1	1					

* Inoculum—85,300 cells per mouse.

† Calculated probits obtained from the fitted maximum likelihood dose response curve. A 3-way common probit slope curve was fitted with the use of two cycles of the Cornfield-Mantel procedure (4).

‡ Two mice apparently tumor-free at 90 days.

§ One mouse apparently tumor-free at 90 days.

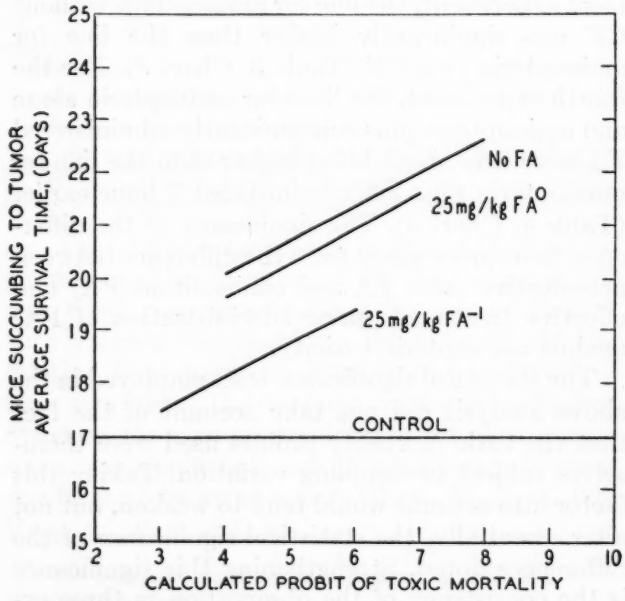

CHART 4 (Experiment 4).—Fitted least squares relationships between time to death from tumor for mice surviving aminopterin toxicity and the calculated probit of toxic mortality from aminopterin. Separate common slope lines are shown for 0 FA, concomitant FA, and for FA injected 1 hour prior to aminopterin. Lines are drawn to extend approximately over the range of calculated probits at which there were any survivors. Control line is based on the average tumor survival time for 30 mice receiving no aminopterin.

administration of FA reduces aminopterin toxicity in a manner similar to concomitant administration of CF (8). The observation that aminopterin is more effective against the tumor than FA plus aminopterin, when the FA is administered 1 hour prior to aminopterin, provides additional experimental consistency. In further support of this finding, all six mice which survived in the four experiments and were apparently tumor-free for the period of observation[8] did not receive effective antitoxicity metabolite treatment. These six mice were among the 168 survivors of aminopterin which received either no metabolite or only concomitant FA. There were no tumor-free survivors among the 278 aminopterin-toxicity survivors treated with CF or 1 hour prior with FA. All the 126 control mice receiving no aminopterin succumbed to the growth of the tumor.

DISCUSSION

These experiments indicate that the antileukemic action of aminopterin, as well as its toxicity to the host, is influenced by the relative doses of anti-

[8] These mice were reinoculated with Leukemia L 1210 after a 90-day observation period. Palpable tumors appeared at the reinoculation site and the mice succumbed in approximately 2 weeks.

metabolite and metabolite. The general similarities of effect on tumor and host were as follows:

a) As the dose of aminopterin was increased, progressive increases were noted in both the number of animals that succumbed from drug toxicity and the survival time of those animals that survived drug toxicity and ultimately succumbed to the growth of the tumor. This occurred not only with aminopterin alone, but also when CF was administered concomitantly with aminopterin and when FA was administered prior to or concomitantly with aminopterin.

b) When CF was administered concomitantly with aminopterin or when FA was administered 1 hour prior to aminopterin, the host-toxicity of a specified dose of aminopterin and its effectiveness against the tumor were reduced.

c) When FA was administered concomitanty with aminopterin, there was no alteration of host or tumor response to aminopterin.

However, the effect of the antimetabolite-metabolite ratio on the tumor did not entirely parallel the effect on the host. The toxicity to mice resulting from administration of aminopterin concomitantly with varying levels of CF is consistent with the modified probit plane model $Y = a + b$ log (aminopterin/[C + CF]), so that for any specified probit of per cent mortality a linear relationship obtains between the dose of aminopterin and the dose of concomitantly administered CF (aminopterin/[C + CF] = K_p)[9] (9). If the effect of the antimetabolite-metabolite ratio on the tumor paralleled the effect on the host, the average day of death would be the same at an equal probit of per cent mortality, and the regression lines relating day of death from tumor to probit of per cent mortality would be the same for all levels of CF employed. However, this did not occur. For equivalent probit of per cent mortality, the animals which survived treatment, with aminopterin plus concomitantly administered CF, showed a lesser increase in survival time with tumor than animals treated with aminopterin alone. A similar result was obtained on administration of FA 1 hour prior to aminopterin. The latter observation is consistent with the evidence that FA, on prior administration to aminopterin, acts as a precursor of CF (1, 8, 10, 15, 16).

It has not been determined whether the antitumor action of aminopterin is mediated through the host or whether it acts directly on enzymatic transformations involving FA and CF in the tumor. The difference in response of the tumor at equivalent toxicity for the host would appear to suggest that the tumor is more efficient than the host in utilizing the metabolite to block aminopterin toxicity. This would suggest that the most efficacious procedure to employ in attempting to inhibit tumor growth with aminopterin is one in which the largest reasonably safe dose is administered without metabolite. The metabolite, CF, might be administered following treatment with aminopterin if toxic symptoms occur (17).

The apparently more efficient use by the tumor of the metabolite may not, however, be real. Instead, conceivably, the tumor may have only small endogenous protection against aminopterin, relative to the host's endogenous protection. Under these circumstances, added metabolite would produce a much greater proportionate change in protection for the tumor than for the host. As still further metabolite is added, the proportionate changes for tumor and host would approach equality, giving rise to a plateau effect. The data, as shown in Charts 1 and 2, are consistent with such a model. The closeness of the nonzero CF lines in these figures suggests that such a plateau exists and is reached fairly quickly. Beyond a moderate amount of CF (3.75 mg/kg CF), additional CF provides little further protection to the tumor relative to the protection to the host.

Under this model, administration of aminopterin alone could result in relatively greater, or more rapid, irreversible damage to the tumor than to the host. Injection of CF concomitantly with aminopterin would tend to minimize any such difference in selective effect. This suggests that an effective procedure for tumor treatment would be one in which an ordinarily lethal dose of aminopterin is administered alone but is followed by delayed treatment with a massive dose of CF. This would permit the aminopterin to selectively damage the tumor, while the added CF is made available to the host before extensive damage to the host has occurred.[10]

SUMMARY

A comparison has been made, with reference to drug dose mortality response, of the relative effectiveness of the following treatments in influencing the survival time of DBA male mice inoculated with a lymphoid leukemia: (*a*) aminopterin; (*b*) aminopterin plus concomitantly administered citrovorum factor; (*c*) aminopterin plus folic acid; where the folic acid was administered 1 hour early; (*d*) aminopterin plus concomitantly administered folic acid. To provide a basis for the analyses employed, the experiments, involving single dose administration of antimetabolite and metabolite,

[9] K_p is the slope of the aminopterin-CF line for percentage mortality p (9).

[10] Experimental results which support this alternative explanation of the selective action of aminopterin were presented in part at the Forty-fourth Annual Meeting of the American Association for Cancer Research, Chicago, Illinois, April 9–11, 1953.

were designed to result in a temporal separation of drug mortality and tumor mortality.

With all the treatments employed, as the dose of aminopterin was increased there was a progressive increase of both drug mortality and survival time of animals which succumbed to the leukemia.

On concomitant administration of citrovorum factor or prior administration of folic acid, with a specified dose of aminopterin, both the mortality response and the increase in survival time ordinarily resulting from treatment with that dose of aminopterin were reduced. For equivalent cost in drug dose mortality, aminopterin was more effective in increasing the survival time of animals which succumbed to the leukemia than aminopterin plus concomitantly administered CF or aminopterin plus FA administered early.

On simultaneous administration of FA with aminopterin, the FA did not alter the toxicity to the host or influence the antileukemic action of the aminopterin.

These results are discussed with reference to the mode of action of aminopterin and the possible implications for therapy.

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Amino Acid Analysis of Serum Proteins in Multiple Myeloma*

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It has long been recognized that the serum proteins of patients with multiple myeloma may differ markedly from those of normal serum as revealed by salt fractionation (11, 22), electrophoretic and ultracentrifugal analysis (3, 16, 19), as well as by other analytical procedures. During the past few years a number of investigators have attempted to determine whether the protein increase in the serum of multiple myeloma patients is simply an increment of normally occurring protein or whether it represents the *de novo* appearance of an abnormal protein (8, 10, 17, 23, 30).

Physico-chemical studies directed toward an understanding of normal and abnormal proteins of plasma and tissues have been conducted in this laboratory (1, 2, 28, 29). As part of this program a study of the proteins of the serum from patients with multiple myeloma was initiated.

Since electrophoresis and ultracentrifuge data for normal gamma globulin are known (9, 16, 20) and since analytical data for the amino acid composition of this protein have been reported (5, 26, 27), it was of interest to conduct similar studies on the myeloma proteins. This was further suggested by the fact that the Bence-Jones proteins, which often appear in the urine of these patients and which have been shown to differ antigenically from normal serum proteins (13), are very low or lacking in methionine (8, 24).

This paper summarizes physico-chemical data for the proteins obtained from the sera of eight different patients with multiple myeloma. It also presents the results of amino acid analyses for these proteins, and compares these values to those for normal gamma globulin.

MATERIALS AND METHODS

Sera used in this study were obtained from hospitalized patients and, in a few cases, at autopsy. The myeloma proteins were separated from the serum as follows: Sera were diluted with 3 volumes of 0.1 M NaCl and dialyzed overnight (2°–3° C.) with stirring against a solution of ammonium sulfate adjusted to pH 7.2 with NH_4OH and of such concentration as to give a final concentration of 1.64 M after equilibration. The precipitates were washed twice with 1.64 M ammonium sulfate and finally dissolved in either 0.15 M NaCl or 0.1 M phosphate buffer, pH 8.0. This method was effective for the preparation of proteins with mobilities in the range of β-globulins as well as for the slower ones, although protein 2 in this group was difficult to keep in solution once it had been separated from the other serum constituents. The precipitated proteins migrated essentially as single components (91–97 per cent) in the electrophoresis cell.

Protein nitrogen was determined by the standard semimicro-Kjeldahl technic.

Electrophoresis experiments were carried out under conditions described previously (7) in pH 8.6 barbital buffer of ionic strength 0.1. Protein concentrations were adjusted to 2 per cent for sera and to 1 per cent for the different precipitated fractions.

One per cent solutions of the proteins were analyzed in the Spinco analytical ultracentrifuge. Samples were prepared for analysis by dialyzing with stirring for 12 hours against a 0.001 M phosphate, 0.15 M NaCl solution at pH 7.4. Sedimentation constants have been corrected to water at 20° C., and are reported for the 1 per cent solutions.

The microbiological assay method described by Henderson and Snell (14) was used in quantitating the amino acid composition of the proteins. Acid hydrolysates were used for the assay of all the amino acids with the exception of tryptophan, in which case alkaline hydrolysates were employed. Both acid and alkaline hydrolysates were used in the estimation of tyrosine. Hydrolysis was effected by autoclaving 0.5 gm. of protein with 20 ml. of 3 N HCl or with 10 ml. of 5 N NaOH, for 10

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and 15 hours, respectively, at 15 pounds pressure. The samples, in Erlenmeyer flasks, were covered with an inverted beaker during autoclaving.

Assay cultures were carried in a total volume of 2 ml. Lactic acid production was measured by titration with 0.02 N or 0.04 N NaOH. Each value reported represents several separate assays (3–6), each of which was carried out in triplicate at five concentrations. Assays usually agreed within better than 5 per cent, and in no case was the variation greater than 10 per cent. Repeat assays were usually on the same hydrolysate, due to the limited amounts of myeloma proteins available.

RESULTS

The electrophoresis and ultracentrifuge studies are summarized in Table 1. It is clear that there is no apparent correlation between the degree of hyperproteinemia, the concentration of the abnormal protein, the electrophoretic mobility, and the sedimentation constant of the abnormal protein.

It can be seen from Table 2 that there is considerable variation in amino acid composition among the several myeloma proteins. Protein 2 differs from the others in its content of several amino acids, arginine being increased, while tyrosine, leucine, and lysine are decreased. This is of particular interest, since this is also the protein which was so difficult to keep in solution and which had a main component with a sedimentation constant of 17.7 *S* as noted in Table 1. Similar properties have been described for part of the γ-1 protein separated from normal plasma pools by Deutsch *et al.* (9).

Although, for purposes of comparison, the ultracentrifuge determinations were made on protein solutions of 1 per cent concentrations, several dilutions of proteins 1 and 2 were analyzed to determine the effect of concentration on the sedimentation of these proteins. Extrapolated to zero concentration, the corrected sedimentation constant S^0_{20} for 1 was 6.86×10^{-13}, and for the main component of 2, 19.2×10^{-13}.

TABLE 1

ELECTROPHORETIC MOBILITY AND SEDIMENTATION CONSTANTS OF MYELOMA PROTEINS

Serum	Total protein (gm/100 ml)	Per cent abnormal protein of total serum proteins	Isolated myeloma proteins: Mobility* (×cm²/v sec×10⁻⁵)	S_{20w}			
1	10.8	52.9	1.80	6.26			
2	7.5	60.0	1.31	7.18	17.7†	26.2	
3	12.5	67.2	1.19	6.37			
4	11.8	47.7	1.12	6.33			
5	8.4	65.1	2.99	6.10†	8.5	10.4	12.1
6	11.3	61.6	1.41	6.20†	8.4		
7	8.7	53.3	2.97	6.28†	8.9		
8	13.0	65.8	1.56	6.35†	9.6		

* Under the same conditions the mobility of normal γ-globulin is 1.49 cm²/v sec × 10^{-5}.
† Denotes the main component.

TABLE 2

COMPARISON OF THE AMINO ACID COMPOSITION OF MYELOMA PROTEINS AND NORMAL γ-GLOBULIN

	Myeloma proteins isolated from different sera								γ-globulin (5)
	1	2	3	4	5	6	7	8	
	(gm/100 gm protein*)								
Arginine	4.4	6.5	4.3	4.7	5.1	4.3	4.8	5.2	4.8
Aspartic	10.6	9.4	9.3	9.9	9.0	7.5	8.7	9.4	8.8
Glutamic	11.7	11.6	10.5	12.0	12.0	8.8	11.5	11.6	11.8
Glycine	5.3	5.6	4.5	4.6	5.9	5.4	5.4	4.9	4.2
Histidine	2.6	2.2	3.0	2.8	2.8	2.9	2.8	3.6	2.5
Isoleucine	3.4	3.4	2.1	2.9	1.7	3.3	4.0	3.2	2.7
Leucine	8.7	7.2	8.3	8.4	10.7	8.6	9.1	9.3	9.3
Lysine	10.4	7.8	9.6	9.5	9.2	8.9	9.7	9.9	8.1
Methionine	1.6	1.7	1.0	1.2	1.0	0.73	0.96	0.54	1.1
Phenylalanine	5.8	5.6	3.9	5.2	4.8	4.9	4.1	5.7	4.6
Proline	8.7	9.7	9.6	8.2	9.4	10.1	10.1	8.7	8.1
Serine	11.6	11.1	11.7	11.6	9.5	13.2	12.4	13.2	11.4
Threonine	7.3	8.4	8.4	7.1	8.7	9.5	9.5	7.9	8.4
Tryptophan	2.2	1.7	2.3	2.3	2.9	2.8	2.4	1.9	2.9
Tyrosine	6.4	3.8	5.6	5.6	4.9	6.6	6.2	6.3	6.8
Valine	8.7	8.9	9.3	9.3	8.0	9.7	8.5	9.1	9.7

* Protein concentration based on N × 6.25.

If histograms are constructed for the amino acid composition of the myeloma proteins, γ-globulin, and other purified proteins available in the literature, it is apparent that the over-all patterns for the myeloma proteins, including those with the mobility of β-globulin, show striking similarity to that of normal γ-globulin.

On the other hand, the variations in composition noted may be indicative of small differences in the composition of the proteins, which are further revealed by differences in electrophoretic mobility, as has been reported by Smith and co-workers (26, 27) for two fractions of normal human γ-globulin.

DISCUSSION

Although there has been no attempt to correlate clinical observations with the analytical data in the present study, work from other laboratories indicates that, to the present time, it has not been possible to predict the type of protein abnormality, whether of the serum or urinary proteins, or both, on the basis of clinical findings.

In relating these proteins which appear in the serum to the urinary Bence-Jones proteins, in which methionine has been shown to be present in low concentrations or absent, it is significant that the serum myeloma proteins all showed the presence of methionine. Whether the presence of methionine bears any relation to the suggestion that the Bence-Jones proteins may be split-products of the myeloma serum proteins (25), or whether it may be related to a series of events in the synthesis of these proteins, remains to be determined. A recent report (12) of an experiment in which labeled glycine was given to a patient with multiple myeloma, however, indicates not only that the half-life times of the abnormal plasma protein and the urinary Bence-Jones proteins are different but that their rates of synthesis are independent.

It is difficult to assess the significance of the differences in amino acid composition, both among the myeloma proteins and between the latter and normal γ-globulin, in terms of whether or not the proteins are normal globulins. It has been demonstrated (6, 9) that normal γ-globulin is, in fact, made up of a number of proteins. In view of this, it might be expected that constituent proteins with a narrow mobility range would vary somewhat in their amino acid content when compared to γ-globulin. There is a good possibility that the myeloma proteins are indeed the same as some of the normally occurring serum proteins. This possibility is supported by the fact that all the sedimentation constants found for the myeloma proteins in the present study have also been reported for normal γ-globulin preparations (6, 9, 15). Immunologic experiments on this point have been conflicting, but, using improved technics, Kunkel *et al.* (17) have demonstrated that some myeloma proteins are related to at least a part of the normal γ-globulin; and Wunderly *et al.* (31) have recently reported that their results do not justify assuming the presence of atypical proteins. If it is true, as indicated by a number of reports (4, 18, 21, 23), that the myeloma proteins are being produced by the malignant plasma cells, it seems reasonable that small amounts of these same proteins could be formed by the smaller number of plasma cells normally found. To this end it would be desirable to have more fractions of normal γ-globulin in order to compare their amino acid compositions to those of the different myeloma proteins.

SUMMARY

Eight proteins have been separated by ammonium sulfate precipitation from the sera of different patients with multiple myeloma. The electrophoretic and ultracentrifugal properties of these proteins are summarized.

Analytical data for the amino acid composition of these proteins indicate differences among them, especially for one relatively insoluble protein which differed markedly from the rest in that it had an increased arginine and decreased tyrosine, leucine, and lysine concentrations. It was further distinguished by having as its main component a protein with a sedimentation constant (S^0_{20}) of 19.2. In a comparison with the amino acid composition of normal gamma globulins, the myeloma proteins were in general high in lysine and proline and low in valine. The over-all similarity of the amino acid composition of these proteins to normal γ-globulin is discussed.

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Experimental Production of Carcinoma with Cigarette Tar*

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The increasing frequency of primary cancer of the lung in many parts of the world has aroused great interest in this condition and has stimulated a search for an explanation. In 1950, Wynder and Graham (40), on the basis of a clinical and statistical investigation, presented evidence of a real association between lung cancer and smoking, especially of cigarettes. These data have been well substantiated by a large-scale British study by Doll and Hill (9, 10). Both studies showed that the risk of developing cancer of the lung increases in direct proportion to the amount of smoking. Ten other recent studies reached similar conclusions (3, 11, 15, 20, 21, 24, 26, 33, 34, 42). In 1952, The Council of International Organizations of Medical Sciences convened a symposium on the endemiology of lung cancer and agreed that the present evidence points to a relationship between lung cancer and cigarette smoking (12).

Tobacco is also thought to play some role in the production of cancer of the larynx, oral cavity, and esophagus. Although the studies of those relationships are not so complete as the studies on lung cancer, the collected data are suggestive (33, 41).

The increasing incidence of bronchiogenic carcinoma and the available evidence relating smoking to it and possibly to cancer of other sites led us to undertake the experimental work reported here. This investigation is directed toward determining in laboratory animals whether there are carcinogenic factors in cigarette smoke.

PREVIOUS INVESTIGATIONS

Many attempts have been made with tobacco products to induce cancers in a variety of experimental animals. The first was reported by Brosch in 1900 (4). He painted guinea pigs with tobacco "juice" for an unknown period of time and described epithelial proliferation. Subsequently, many different approaches to the problem were undertaken with various types of tobacco, different methods of tar preparation, and different species of animals. Many of those studies were carried on for too brief a period of time or with too few animals to be regarded as significant. Hoffmann and his associates (17), for instance, painted animals for only 14 days, at which time they noted hair loss. Wacker and Schmincke (37) observed proliferation of epithelium in rabbits' ears 21 days after a subcutaneous injection of pipe tar.

The first recorded experiment with mice and with tobacco tars as the suspected carcinogen was the one just cited by Hoffmann and co-workers. The more detailed of the subsequent studies are listed in Table 1. This table attempts to summarize the methods used in the various studies and the results obtained. In many instances the method of study was not described in sufficient detail to give all the information considered essential.

From this survey of the literature it is found that, before our study, all the previous attempts to produce experimental cancer in mice with tobacco products were successful in the production of only seven epidermoid cancers of the skin.

Several investigators attempted to induce pulmonary tumors in mice with tobacco smoke. Lorenz and co-workers (22) obtained negative results in this manner. Campbell (5), and especially Essenberg (13), however, claim to have found a significantly higher percentage of pulmonary adenomas in the experimental than in the control group. It is doubtful that such a finding is important. At any rate, so far these methods have not induced true bronchiogenic carcinomas.

The majority of the investigators working with tobacco tars used rabbits as the experimental animals (14, 23, 27–32, 36). In view of the fact that the present work deals with mice we shall only briefly list some of the studies with rabbits. Roffo reported the production of carcinomas in rabbit ears after painting the ears with a distillate of tobacco (28, 30, 32). Sugiura (36), in attempting

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TABLE 1. SUMMARY OF METHODOLOGY OF SOME STUDIES ON TOBACCO TAR AS A POSSIBLE CARCINOGEN FOR MICE

Author	Type of Tobacco	Method of Tar Collection	Temperature	Portion of Tar Used	Number of Mice	Solvent
Helwig (16), 1928	Not given	Tarry extract from bowls of burned briar pipes	Unknown	Denicotinized tar	50	Ether with olive oil
	Not given	Distillate produced at temperatures of 400°–500° C. and tarry product extracted with ether or chloroform	400°–500° C.	Extracts	50	Chloroform or ether
Bogen and Loomis (2), 1932	Kentucky Burley tobacco	Destructive distillation and extraction with H_2SO_4, alcohol, and ether	Not given	50 per cent whole tar solution in glycerine	12	Glycerine
Cooper (7), 1932	Not given	Intermittently puffed and exhaled pipe tobacco; the resulting tar condensate was then distilled	370°–590° C.; 480°–700° C.	I Tobacco tar of 400°–500° C.—wooden pipes		Alcohol, benzene, and glycerol
				A. 10 per cent in alcohol solution	4	
				B. 30 per cent solution in glycerol	6	
				C. Neutral fraction freed from bases or phenols using either		
				1. 50 per cent solution in benzene	13	
				2. 10 per cent solution in glycerol		
				D. Original tar dissolved in glycerol and applied hot at 55° C.	6	
				II Tobacco tar of 700°–800° C.—wooden pipes; 25 per cent solution in alcohol	9	
				III Tobacco tar of 400°–500° C.—clay pipes; 50 per cent solution in alcohol	12	
Schuerch and Winterstein (35), 1935	Cigars	Smoke of cigars collected in cotton which was extracted with chloroform. The solution was denicotinized with 2 per cent HCl	Not given	I 25 per cent tar in alcohol solution	100	Alcohol
				II Tar with nicotine applied for 3 months then used denicotinized tar	100	
				III Denicotinized tar extracted with chloroform	100	Chloroform
				IV Denicotinized tar extract heated to 130° C. prior to painting	100	
				V Denicotinized tar	100	
				VI Tar extract distilled at 100° C.	100	
				VII Tar extract distilled at 160°–170° C.	100	
				VIII Tar extract distilled at 180° C.	100	
				IX Tar extract distilled at 300° C.	100	
Taki (19), 1937	Unknown	Extract from tobacco pipes dissolved in ether and filtered, then denicotinized with HCl	Not given	Denicotinized tar	104	Ether
Campbell (6), 1939	Not given	Tar condensate (partial) of artificially smoked cigarettes	Not given	Tar condensate	23	None
Sugiura (36), 1940	Mixture of American and Turkish leaf tobaccos	Distillation at 100°–500° C. and redistilled at 500°–900° C.	100°–900° C.	I A. Distillates applied for 90 days	186	None
				B. After 90 days		
				1. Watery fluid of first distillate	44	
				2. Oily liquid of first distillate	57	
				3. Second distillate, 500°–900° C.	67	
				C. Cholesterol diet during painting	40	
				II Oily liquid of first and second distillates	30 C57 30 DBA	
				III Control animals using coal tar	40	
Flory (14), 1941	Entire leaf and stem of cured Kentucky tobacco	Destructive distillation at 700° C. for 6–8 hours yielding 3 fractions	120°–700° C.	I 350°–700° C. destructive distillate	30	None
				II Denicotinized 350°–700° C. destructive distillate	46	
				III Denicotinized 120°–350° C. destructive distillate	76	
				IV Mixture of watery parts of distillates	30	
				V Control group; coal tar and benzene in equal parts	15	
	Coarsely ground tobacco	Continuous suction applied to clay pipes at 520°–730° C.	520°–730° C.	Denicotinized whole pipe tar	60	None
Shubik,† 1950	Cigarettes	Tar extracted with acetone from smoked cigarette stubs	Not given	I Tar extract	30	Acetone
				II Tar extract applied for a limited period followed by applications of croton oil for duration of experiment	30	
Wynder, Graham, and Croninger, 1953	Cigarettes	Whole tarry condensate of intermittently puffed cigarettes	Burning tip—316°–599° C.; smoke—26.7°–60.0° C.; combustion—966° C. (max.), 682° C. (av.)	I 50 per cent whole tar in acetone		U.S.P. acetone; white mineral oil (used with croton oil)
				1. Tar	81	
				2. Tar/croton	31	
				II Acetone controls		
				1. Acetone	30	
				2. Acetone/croton	14	
				III 0.3 per cent methylcholanthrene	25	

* Applied by painting the skin

† P. Shubik, personal communication.

TABLE 1—*Continued*

Strain and Initial Age of Application	Frequency and Dosage of Application*	Duration of Applications	Results	Author
Not given	3 times a week; dosage not given	1 yr.	Ulceration	Helwig (16), 1928
Not given	Not given	8 mos.	Ulceration	
Not given	2 times a week; dosage not given	Over 1 yr.	Negative	Bogen and Loomis (2), 1932
Not given	3 day intervals; dosage not given	Over 16 mos.	One epithelioma from 10 per cent tar (produced at 400°–500° C.) in alcohol solution in 16 months	Cooper (7), 1932
			Negative	
			Negative	
Not given	2 times weekly, dosage not given	9 mos.	Atrophy of skin with slight hyperkeratosis and perivascular infiltration	Schuerch and Winterstein (35) 1935
	2 times a week	348 days	Same as I	
	2 times a week	265 days	Same as I	
	2 or 3 times a week	635 days	Atrophy of skin and chronic inflammation	
	2 times a week	270 days	Hair loss	
	Not given	2 mos.	Negative	
	3 times a week	190 days	Loss of hair	
	Not given	6 mos.	Loss of hair and atrophy of skin	
	Not given	480 days	Atrophy of skin and hair loss	
Not given	2 times a week; dosage not given	Not given	3 papillomas (1st observed at 85 days); 2 squamous cell epitheliomas	Taki (19), 1937
Not given	2 times a week; dosage not given	20 mos.	One typical epithelioma at 16–20 mos.	Campbell (6), 1939
Bagg albino mice, 2–3 months old	2 times a week; 0.02 gm/dose	90–500 days	A. Negative B. After 90 days 1. No results 2. No results 3. One squamous carcinoma C. No accelerating influence	Sugiura (36), 1940
C57 blacks and DBA mice, age of initial application not given		cholesterol diet—90–180 days C57 black 80–275 days; DBA died too early for results	No tumors	
Bagg albino		75–309 days	75 per cent developed progressively growing tumors with 31 squamous cell carcinomas	
White mice (not an inbred strain); age at initial application not given	5 times a week .015 gm/dose .018 " .015 " .20 "	18 mos.	Papillomas / Cancers 4 in 7½–11½ mos. / 0 8 in 7½–11 mos. / 1 in 8.5 mos. 1 in 14 mos. / 0 0 / 0 13 in 126–256 days	Flory (14), 1941
White mice (not inbred); age at initial application not given	5 times a week; 0.013 gm/painting	18 mos.	2 papillomas in 14 mos. 1 cancer in 17 mos.	
C strain; 3 months	Twice weekly	9 mos.	Negative	Shubik,† 1950
CAF1, 8–12 weeks	Tar: 3 times weekly; 0.040 gm/painting Croton oil: 3 times weekly tar/acetone plus 1 painting/week of 5 per cent croton oil/mineral oil	24 mos. maximum	Papillomas (no.) / Cancers (no.) I Whole tar in acetone (1:1 ratio) 1. Tar 48 / 36 2. Tar/croton 13 / 3 II Acetone controls 1. Acetone 0 / 0 2. Acetone/croton 0 / 0 III 0.3 per cent methylcholanthrene 25 / 25	Wynder, Graham, and Croninger, 1953

to repeat Roffo's work, obtained negative results, whereas Flory (14) in a similar large-scale experiment obtained papillomas and several lesions which he designated as "carcinomatoids" in rabbit ears after application of a tobacco distillate (24, 39).

Roffo also used rats as experimental animals. He implanted pills of tobacco tar into rat bladders and noted cancer-like growths after 8 months (29). McNally (25) applied a water-soluble product of cigarette smoke to the mucous membrane of the oral cavity and to the tongue and skin of rats. All animals were lost within 4 months due to the toxicity of the material, and no significant changes were noted.

MATERIALS AND METHODS

The method of study employed in this investigation is presented in some detail because the varying results of different workers may be a reflection of different methods used. In this study the guiding principle in collecting the tobacco tars was to simulate human smoking habits as closely and as practically as possible. It was felt that any change in temperature or the removal of any fraction from the smoke might alter the effect of the resulting tars. Therefore, we used a smoke condensate obtained from smoking cigarettes intermittently, and, unlike most other investigators, we did not remove the nicotine from the tars in the initial phases of the experiment.

SMOKING APPARATUS

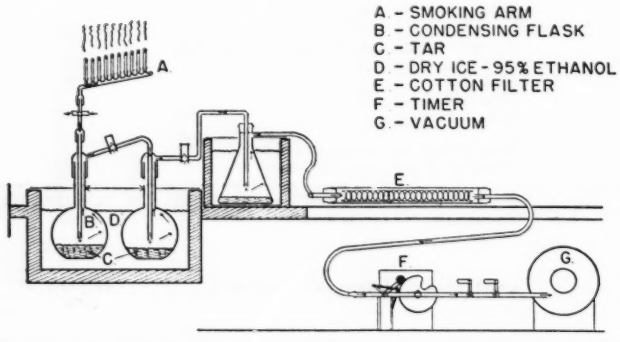

CHART 1.—Schematic drawing of smoking apparatus

Tobacco used.—A popular brand of domestic cigarettes was used.

Smoking apparatus.—The tobacco tars were collected in the following manner. Sixty cigarettes were placed in a battery of glass smoking arms (A) containing separate holders for ten cigarettes each and lighted with a multiple jet gas burner (Chart 1). The smoking arms were rotated and changed as the cigarettes burned down, keeping the apparatus filled with 60 cigarettes at all times after the original loading. The arms were placed in position on glass candelabra-like receivers on each of the two smoking units. These receivers were jointed and connected to a series of three 2,000 ml.-glass flasks (B) and connecting tubes on each unit. A vacuum was created by a small electrically driven air pump (G) (1.3 cu. ft. displacement of free air per minute at a vacuum of 27 in. of mercury). At 18-second intervals an open-shut valve, geared to a three r.p.m. $\frac{1}{4}$ horsepower motor (F), was automatically opened for a 2-second period, allowing the vacuum from the air pump to be applied to the condensing flasks and ultimately to the burning cigarettes. The condensing flasks were enclosed in insulated stainless steel tanks filled with 95 per cent ethanol and dry ice (D) to lower the temperature to approximately −60° C. The smoke that failed to condense inside the cooled tanks then passed on to a third flask outside each unit and insulated with an asbestos jacket. The two units met at a common source of vacuum, at which point a cotton filter (E) was placed in the line to protect the motors from tar corrosion. The smoking time per cigarette averaged from $7\frac{1}{2}$ to 8 minutes or from 28 to 32 puffs per cigarette at a rate of three 2-second puffs per minute. The cigarettes were burned down to approximately a 2–2.5-cm. length.

As the smoke condensed, a dark brown viscous liquid formed and adhered to the glass apparatus with diminishing density to the third set of flasks. After 50 cartons were smoked, all of the tar contained in the glassware above the cotton filter was washed clean with approximately 300 cc. of acetone by dismantling and corking the separate glassware pieces. This tar-acetone mixture was then stored at −7° C. in glass-stoppered bottles until made up into solutions.

Preparation of tars for application.—Solutions were made up fresh monthly from 50 cartons of cigarettes. The choice of acetone as a solvent for these tars was based primarily on the fact that acetone was known to be noncarcinogenic and also because it was found to be the best dissolving medium for this substance.

The tar-acetone mixture was poured into 150-ml. porcelain evaporating dishes, which had been previously weighed; these were placed in an exhaust hood containing a rotating fan for 6 hours and were then left overnight. The average weight of the residual tar obtained in this manner was 9.7 gm/200 cigarettes. An equivalent volume of acetone was added to each gm. of residual tar and this mixture (1 cc. acetone to 1 gm. tar) was placed in 125-ml. Pyrex glass-stoppered bottles and stored at −7° C. until used. When used, small amounts of these solutions were repoured into other bottles to reduce the evaporation of the acetone as much as possible.

Experimental conditions.—Tests for the amount of vacuum applied on the smoking cigarette were made at three stages of burning, i.e., at the start of burning, at midpoint, and at butt stage. In the first stage the magnitude of the vacuum at the start of the puff was zero; it increased to a maximum value of −9 mm. of water and then fell to an average of −8 mm. This value was found to reach a maximum of −7 mm. and an average of −6 mm. in the second stage, and a maximum of −5 mm. with an average of −4 mm. in the butt stage.

Temperatures of the burning cigarette:[1] All the temperature studies were made with iron constantan thermocouples with welded hot junctions. No. 26 gauge duplex glass-insulated wire with a combined resistance of 1.4 ohms/ft at 21.1° C. and a No. 28 gauge duplex enamel insulated wire with a combined resistance of 2.2 ohms/ft at 21.1° C. were used. The temperatures were registered on a suspended galvanometer potentiometer-type null balance, calibrated from 0 to 2,000° F. The burning tip temperatures depended on the penetration of the thermocouple into the combustion zone. The results of 60 temperature readings ranged from 316 to 599° C. The average of the 60 temperature readings was 438° C. with a room temperature of 26.7° C.

Combustion temperatures of the cigarette: Combustion temperatures were obtained by inserting 28 gauge thermocouples up through the mouth end approximately three-quarters of the length of the cigarette and by then smoking the cigarette to the point where the hot junction of the thermocouple would pass through the combustion zone. Tempera-

[1] Temperature studies were carried out by the Fillo Sales & Engineering Co., St. Louis, Mo.

tures up to 966° C. were obtained by this method. The average reading, however, was 682° C.

Temperatures of the smoke as distinguished from that of the burning cigarette: Twenty-eight gauge iron constantan thermocouples were inserted in the receiving end of the cigarette arm, and the cigarettes were smoked down to the short butt stage. No appreciable rise in the temperature of the smoke was noticed until the cigarettes were consumed to butt length. The temperatures of the smoke ranged from 26.7 to 60° C.

Animals used.—One hundred fifty-six CAF_1 mice (genetically homogeneous F_1 hybrids from the cross A/Cloudman inbred strain males by BALB/C inbred strain females obtained from the Roscoe B. Jackson Memorial Laboratory at Bar Harbor, Maine), with an equal distribution of males and females, were started at 8–12 weeks of age.

All the mice were housed in wire-mesh cages containing 12 animals each, and were fed a diet of Rockland mouse pellets, a rolled oat supplement, and water ad libitum. Toward the end of the experiment, animals developing lesions were segregated and placed in individual glass jars.

Subgroups: The CAF_1 mice were originally divided into two groups, i.e., one group of 112 mice painted with tobacco tars in acetone and a second group of 44 mice receiving acetone alone. Preliminary studies on 60 Swiss mice showed that the animals were sensitive to tars containing nicotine, particularly in concentrated solutions. Being cognizant of the sensitivity of mice to tobacco tars, we began the CAF_1 group cautiously using dilute solutions, the concentrations of which were gradually increased. The tarred group was started with tri-weekly paintings of a residual tar/acetone solution containing 1 part of tar to 3 of acetone. The dosages were gradually increased to 1:2 and finally to 1:1 ratio in 2 months; the latter dosage averaged 40 mg of tar/acetone solution per painting.

Prior to each painting the entire dorsal area of each mouse was shaved of excess hair with an electric clipper, and one saturated brushful of the tar/acetone mixture was applied to the shaved area with a No. 5 camel's hair brush; several strokes were used, beginning at the nape of the neck and working backward to the tail region medially and laterally. The control animals were shaved and painted with acetone in the same manner.

Seven months after onset of painting, 31 of the 102 surviving tarred mice and 14 of the 42 surviving controls on acetone were segregated and given an additional painting per week of 1 per cent croton resin in acetone (Berenblum [1]) in order to determine the cocarcinogenic properties of croton resin in relation to tobacco tars. Each subgroup contained an equal number of males and females. The croton resin solution was found to be extremely irritating to the skin of mice and caused widespread ulceration. Dosages were reduced without relief of this condition. Two months later these animals were changed to a 5 per cent solution of croton oil (Croton Oil N. F. VII, Magnus, Mabee, and Reynard, Inc., New York City) in mineral oil (pure Superla No. 34, Standard Oil Co. of Ind.), which, although much less irritating to the skin, seemed to induce severe diarrhea.

In the 15th month of painting a few of the tar/acetone and tar/croton/acetone animals were placed on a denicotinized 1:1 tar/acetone solution, prepared by extracting the nicotine with 1 per cent hydrochloric acid. This treatment was used only when the animal had an open ulcerating lesion. When the lesion scabbed over, the whole tar/acetone solution was reapplied. Whereas most of the animals with lesions were kept on the whole tar/acetone solution throughout the experiment, it was occasionally necessary to resort to this less toxic treatment with denicotinized solutions in order to preserve the animal. Also, as the animals became weakened by old age or disease, the tar paintings were decreased by painting merely the lesion itself. When a lesion had become a grossly positive carcinoma, the tar paintings were stopped completely. The animal was then allowed to live free of paintings and was sacrificed only when death appeared imminent.

RESULTS

General observations.—The whole tar/acetone solution promoted a general thickening of the skin, but rarely produced lasting areas of complete hair loss. The tar/croton group showed more hair loss, thickening, reddening, and proliferation of epithelium than the regular tar group. The acetone/croton group showed hair loss and thickening and scabbing of the skin, but not to so great a degree as those animals on tar/croton. The skin of the acetone controls remained soft, pliable, and normal throughout the experiment.

A difference in the rate of hair regrowth was noted between the experimental and control groups. Tarred animals regrew hair rapidly between paintings and required regular shaving. The tar/croton and acetone/croton animals remained denuded for longer periods, showing spotty and sometimes complete hair loss. The acetone controls always kept a short stubble of hair; yet they regrew hair at a much slower rate than the animals on tar or on croton oil. Histologic sections on a separate group of CAF_1 mice at 10 days showed hyperplasia, and hyperkeratosis in 75 per cent and disappearance of sebaceous glands in 100 per cent of the animals painted with the tar/acetone solution.[2] None of these changes were observed in animals painted with acetone alone.

Little difficulty was encountered because of drug intoxication, since, as described above, the animals' tolerance to nicotine was increased. In one instance, however, unintentional evaporation of a solution apparently led to an increased concentration of nicotine and brought about a number of deaths in the tar/croton oil group. At the onset of the painting procedure, the mice appeared to be in a partially drugged state and exhibited occasional fine tremors following paintings. These tremors and stuporous states were rarely observed after about 2 months of application, but frequently reappeared in old age until the dosages were decreased.

Papilloma formation.—Of 81 tarred CAF_1 mice, 59 per cent—26 females and 22 males—developed papillomas. The earliest appearance was observed during the 33d week, with a mean time of 56 weeks. Of the papillomas, 8.6 per cent regressed. The lesions were uniformly distributed over the painted area. Twenty-three animals had more than one papilloma (Table 2; Chart 2).

A typical papilloma appeared as a firm, low,

[2] Dr. Victoria Suntzeff, personal observation.

TABLE 2

APPEARANCE OF LESIONS IN CAF_1 MICE AFTER PAINTING WITH CONDENSED CIGARETTE TAR, CROTON OIL, AND ACETONE*

GROUP*	No. ALIVE	MOS. OF PAINTING	PAPILLOMAS† (GROSS) No. of mice	CARCINOMAS‡ (GROSS) No. of mice
T	81	1		
T/C§	31	1		
A	30	1		
A/C§	14	1		
T	69	8	3	
T/C	31	8	1	
A	28	8		
A/C	14	8		
T	69	10	8	
T/C	31	10	2	
A	28	10		
A/C	14	10		
T	62	12	15	2
T/C	18	12	3	
A	28	12		
A/C	12	12		
T	50	14	22	2
T/C	8	14	7	
A	23	14		
A/C	11	14		
T	47	16	39	20
T/C	8	16	10	1
A	22	16		
A/C	11	16		
T	41	18	43	25
T/C	7	18	10	1
A	19	18		
A/C	9	18		
T	26	20	48	31
T/C	4	20	11	2
A	18	20		
A/C	7	20		
T	8	22	48	36
T/C	3	22	13	3
A	16	22		
A/C	3	22		
T	0	24	48	36
T/C	0	24	13	3
A	9	24		
A/C	3	24		

* T = tar/acetone, T/C = tar/croton oil/acetone, A = acetone, A/C = acetone/croton oil.

† This column represents cumulative totals on the first gross appearance of papillomas.

‡ This column represents cumulative totals on the first gross appearance of carcinomas, all of which later were verified histologically. In two cases cancer was shown microscopically to be present in tumors that appeared grossly to be papillomas. In these two cases the month of histologic verification was taken as the date of first appearance.

§ Croton oil was started in the seventh month.

broad-based, clearly outlined wart, varying in size from 0.1 to 0.4 cm. in diameter. In most cases these warts were well-defined nodules, occasionally budding before sloughing and showing definite craters.

Cancer formation.—Of 81 tarred mice, 44.4 per cent developed epidermoid cancer of the skin. The first was noted grossly during the forty-second week; the mean time of appearance was 71 weeks. Of 62 mice still alive in the tar group at 12 months, the time at which the first gross cancer was observed, 58 per cent developed cancer.

Twenty-five of the carcinomas occurred in females and 11 in male mice. The females, although their average weight was about 4 grams less than that of the males, had a longer survival time in the tar group than the males. Of 41 male and 40 female mice started on experiment, at 12 months 34 female and only 28 male mice were alive. At 18 months the respective figures were 26 and 19. In the control group twice as many males lived for 18 months as females.

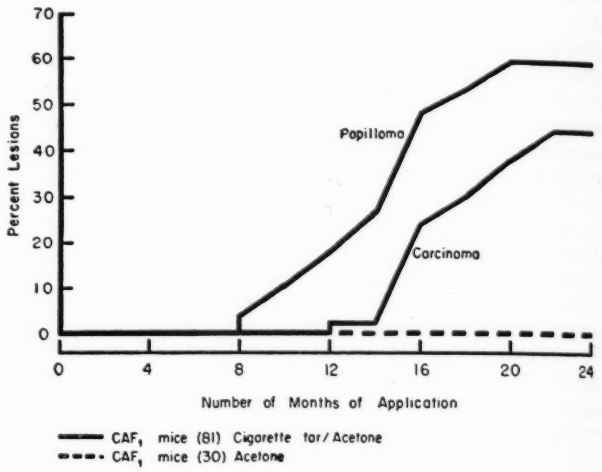

CHART 2.—Month of first gross appearance of papillomas and carcinomas in CAF_1 mice painted with a cigarette tar/acetone solution, and a group of CAF_1 mice painted with acetone alone. All of these lesions were subsequently proved histologically. In two instances, where a cancer was found in a tumor which appeared grossly to be a papilloma, the month of death was taken as the time of first appearance of the cancer.

Of the cancers, the majority—27 (75 per cent)—had previous papillomatous growths; however, a number arose from localized ulcerating sites. Two cancers, which had been classified as papillomas at autopsy, were discovered upon histological examination. The cancers were always found only on the painted area, and in advanced cases often spread laterally beyond the area of painting. Six mice had two separate skin cancers. Prior to the development of the typical cancer, a localized thickening and ridging of the epidermis surrounding the papilloma in the shape of a rolled, puckering rim was noted. During this process of initial invasion, the papilloma proper sloughed off and left a small ulcerating crater. These craters widened and continued to ulcerate. They frequently became necrotic and grossly infected. The rims and bases also became more fully cratered and hardened. In

the advanced stages the cancers appeared as grossly massive ulcers with definite rims (Figs. 1, 3, 5, 7, 8, 10, 11, 13, 14, 16, 18, 20). Histological examination of these lesions showed the usual characteristics of highly malignant epidermoid skin cancer —downward invasion of epithelial cells with extension through the basement membrane, lack of differentiation, and many typical mitotic figures (Figs. 2, 4, 9, 12, 15, 17).

Tar/croton oil.—The tar/croton oil group was begun in the 7th month after the onset of tar application. Of 31 mice segregated into this group, 42 per cent developed papillomas and 9.7 per cent carcinomas. The lesions did not appear earlier than in the tarred group. The first papilloma was noted in the 8th month, and the first cancer in the 16th month.

It is not possible to evaluate these data because 77 per cent of the animals died between the 12th and 14th months, subsequent to marked weight loss. On one occasion, one group of twelve animals was thought to have received an overdose of tar, resulting from excessive acetone evaporation. This group of animals died after having convulsions.

Acetone group.—Thirty animals comprised this group. Nine—30 per cent—were still living at the end of 24 months, in contrast to 0 per cent in the tarred group. No lesions were noted in this group of mice. The epidermis remained soft and pliable throughout the experiment. The average weight of animals in this group through most of the study was about 1 gm. more than in the tarred mice, although, at the onset of the experiment, the average weight of the mice in the tarred group was 2 gm. greater than in the acetone control group.

Acetone/croton oil group.—A group of fourteen mice originally treated only with acetone received an additional painting of croton oil per week starting at the 7th month. In this group three mice—21 per cent—were still living at 24 months. The epidermis became roughened and thickened following repeated applications of croton oil. No papillomas or carcinomas appeared in this group.

Methylcholanthrene group.—Twenty-five CAF_1 mice were painted with a solution of 0.3 per cent methylcholanthrene in acetone 3 times a week. They all developed carcinomas within $4\frac{1}{2}$ months. The first papilloma was noted during the 6th week, and their average time of appearance was 7 weeks. The respective figures for carcinoma formation were 12 and 16 weeks.

Transplantation.—Two of the cancers in the tarred group have been successfully transplanted. One cancer was transplanted for 4 generations by Dr. V. Suntzeff before it became grossly infected. The method of transplantation was as follows: The tumor was excised from the living host, and small healthy pieces were cut and placed in either a penicillin-streptomycin solution or a saline solution before being transplanted subcutaneously with a sterile trocar to the lateral chest region. The other cancer was transplanted in our laboratory directly from the living animal to another CAF_1 mouse with a sterile trocar without first placing the tumor into a solution. The host and the mouse to receive the tumor were previously injected subcutaneously with 100 units of dihydrocillin;[3] this dose was repeated for 3 days after transplantation. For every third generation since the sixth generation, the tumor has been excised, minced, and soaked in either dihydrocillin or syncrobin[4] for 15–45 minutes prior to transplantation as a protective measure against infection; subcutaneous injections of antibiotic have been omitted. Preliminary data on the rate of growth of this tumor show that transplants without the intermediary soaking in antibiotic grow to approximately 0.5–1 cm. in 15–21 days before ulcerating and being retransplanted, whereas transplants previously soaked in antibiotic grow more slowly ($\frac{3}{4}$–1 cm. in size in 29–43 days). This tumor is still an active epidermoid carcinoma in the 13th generation with 100 per cent takes (Figs. 6 and 19).

DISCUSSION

Evaluation of method of present study.—The basic principle in tar collection was to simulate human smoking habits as closely and as practically as possible. A popular brand of cigarettes was used. The temperature factors were found to be similar to those encountered in human smoking. The frequency of smoking (3 times a minute) is greater than that encountered in the average human smoking. In view of the fact that this increase involved no significant temperature changes, the more frequent puffing rate was chosen for the practical purpose of obtaining the required amount of tar more rapidly and economically.

Since we assumed that the carcinogenic effect of tobacco tars might be owing to a summation effect of subthreshold carcinogens or that such a factor might be in the alkaloid fraction of the tars, the whole condensate was used. Acetone, an established noncarcinogen, was found to be the best solvent for the tobacco tar and, therefore, the tar was administered as a tar/acetone solution.

[3] Combination of streptomycin and penicillin by Upjohn & Co.

[4] Combination of streptomycin and penicillin by Schenley, Inc.

We postulated that tobacco tars have only a weak carcinogenic activity and thus would exhibit a long latent period. Therefore, we started with 8–12-week old mice. Our results clearly indicate that if all the mice had died during the first year of painting or if we had stopped the experiment at that time, the data would have been much less significant. For the same reason, we used a higher dosage schedule than had been used by previous investigators. Subsequent studies may show that an additional increase in dosage may further accelerate tumor formation.

Preliminary studies indicated some difficulties with the tar solution that contained nicotine. This problem could be overcome, however, with the desensitization procedure employed.

The basic factors of the method, therefore, were to collect the tar in a manner simulating human smoking habits, to use the whole tar obtained, and to apply it in relatively large quantitites and for a long time. This approach may be in part responsible for the greater production of cancer in this investigation compared to that of previous studies. The possibility remains that the CAF_1 strain of mice may be particularly susceptible to the carcinogenic effect of cigarette tars.

Effect of croton oil.—Subgroups were painted with croton oil to determine whether tumor formation could be speeded up, as had been indicated by Berenblum (1). The CAF_1 mice received croton oil in mineral oil once a week starting with the 7th month after the painting with tobacco tars was begun. Because of a high mortality during the 12th and 14th months in this group, the croton-oil effect on tumor formation cannot be properly evaluated from this experiment, although within the period of observation no acceleration of cancer formation was noted.

Tobacco versus methylcholanthrene.—Although this study establishes condensed cigarette smoke as a carcinogen for mouse epidermis, its activity is less potent than that of methylcholanthrene. Methylcholanthrene, of course, is a very potent carcinogen, rather than a crude substance in which suspected carcinogens may be diluted.

Although crude tobacco tar is less potent as a carcinogen than methylcholanthrene for mouse epidermis on the basis of the number of cancers induced and the length of the latent period, the fact remains that over 40 per cent of the animals painted with tar from cigarette smoke developed carcinomas, whereas no lesions were observed in the control animals.

Animal versus human data.—Animal data do not necessarily confirm or deny human data, although historically much of our present understanding of carcinogenesis is based on corollary studies between clinical and laboratory research. The studies on coal tars from Pott to Yamagiwa and Ichikawa and to Cook, Hieger, and Kennaway serve as a classical example. In coal tar investigations experimental data confirmed the clinical data, and thus added import was given to both.

A similar relationship now exists in the tobacco tar field. Here, too, a clinical association between smoking and cancer seems established. It has been shown that a condensate of this smoke may induce epidermoid cancer of the skin in the experimental animal. The suspected human carcinogen has thus been proved to be a carcinogen for a laboratory animal.

The most far-reaching effect of this observation, perhaps, does not lie in its immediate relationship to human carcinogenesis, but, rather, in that the proven susceptibility of animals furnishes us with a working tool which may enable us to identify and isolate the carcinogenic agent(s) within the tars.

It is an interesting and perhaps significant fact that with the CAF_1 mice it was necessary to have an exposure to the tar for approximately one-half of the life span of the animals (average 71 weeks). This agrees roughly with the previous finding of Wynder and Graham (40) that the maximum incidence of bronchiogenic carcinoma in the human occurs after 30–35 years of smoking—about one-half of the human life span.

Tobacco as a carcinogen.—It appears surprising that, in view of the detailed work carried on with coal tars and its derivatives, relatively little work has been done with tobacco tars. Because recent clinical data have placed increasing emphasis on tobacco as a human carcinogen, it is our hope that more investigators may approach the tobacco-cancer problem.

Although several hundred chemical and physical agents have been found to induce cancer in animals, each one of these agents seems to have a specific carcinogenic action. Chronic traumatic irritation as such does not induce cancer to any significant degree. Therefore, in view of the positive animal and human data, we suspect that tobacco contains specific carcinogen(s).

Which fraction of the tar is carcinogenic is not yet known. Roffo (31) claimed to have identified benzpyrene in tobacco tar, but this could not be confirmed by Hirst and his co-workers (7), and more recently that substance could not be detected by Waller (38). An examination by Eby[5] of the tobacco tar used in this study did not reveal any spectroscopic evidence of the known carcinogenic aromatic hydrocarbons. Arsenic, an accepted hu-

[5] J. Eby, personal communication.

man carcinogen, is present in tobacco, but recent studies by Daff and co-workers (8) based on the arsenic content of various types of European tobacco tends to place less emphasis on this inorganic element. Heat, cigarette paper, flavoring, and wetting agents have been suggested as etiologic factors in the production of cancer, but it must be noted that clinical evidence has also pointed to cigar-smoking, pipe-smoking, and tobacco-chewing as possible factors in the production of cancer of the respiratory and alimentary tract.

The actual carcinogenic agent or agents in tobacco remain to be identified. Studies combining chemical and biologic efforts leading to their identification are urgently needed. Should one be able to identify definite carcinogens and succeed in removing them, or at least in reducing their quantity in tobacco, proper preventive methods would be at hand. Such studies may further our understanding of human and animal carcinogenesis and may lead to the development of practical preventive measures against cancer.

SUMMARY AND CONCLUSIONS

1. A cigarette tar condensate was obtained with a smoking machine which simulated human smoking habits. The resulting tar was dissolved in acetone and applied to the backs of CAF_1 mice in a dosage of 40 mg. of tar/acetone solution 3 times a week. Control mice were painted with acetone.

2. Of 81 tarred mice, 59 per cent developed papillomas. The first lesion was noted in the 33d week, and the mean time of appearance was 56 weeks.

3. Of 81 tarred mice, 44 per cent developed histologically proved carcinomas. The first carcinoma was observed in the 42d week, and the average time of appearance was 71 weeks. Of 62 mice alive at 12 months, 58 per cent developed cancer. Seventy-one weeks constitutes approximately one-half of the life span of CAF_1 mice. This corresponds roughly with the fact already noted that in the human about 30–35 years of smoking, or approximately one-half the life span, are required for the production of bronchiogenic carcinoma.

4. One carcinoma was transplanted for 4 generations and another one is currently growing in the 13th generation.

5. Control mice painted with acetone alone showed no skin lesions. At the end of 20 months of painting, 53 per cent were still living, compared to 9.8 per cent in the group painted with tobacco tars.

6. The group of mice painted with croton oil in addition to the tar, starting in the 7th month, cannot be properly evaluated because of a greater number of deaths occurring during the 12th and 14th months, although within the period of observation no acceleration of cancer formation was noted.

7. The group of mice started with acetone and receiving croton oil beginning in the 7th month showed roughening and thickening of the epidermis, but no tumor formation was noted.

8. All CAF_1 mice painted with 0.3 per cent solution of methylcholanthrene in acetone developed cancer within $4\frac{1}{2}$ months. The first papilloma appeared during the 6th week, with average appearance during the 7th seek. The first carcinoma was observed during the 12th week, with a mean time of appearance of 16 weeks.

9. The results obtained with CAF_1 mice establish condensed cigarette tar as a carcinogen for mouse epidermis. These studies provide a tool to determine and isolate the possible carcinogenic agent(s) within tobacco tar. At present it is not known which fraction or fractions in tobacco tars are carcinogenic. Combined chemical and biologic studies are now in progress to search for such agents. Such studies, in view of the corollary clinical data relating smoking to various types of cancer, appear urgent. They may result not only in furthering our knowledge of carcinogenesis, but in promoting some practical aspects of cancer prevention.

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Fig. 1.—No. 87 CAF_1. 322 days. Beginning carcinoma.

Fig. 2.—Photomicrograph of carcinoma. No. 87 CAF_1 at 371 days. ×52.

Fig. 3.—No. 87 CAF_1. 371 days. Advanced carcinoma.

Fig. 4.—Photomicrograph of carcinoma. No. 87 CAF_1 at 371 days. ×215.

Fig. 5.—No. 87 CAF_1. 371 days. Advanced carcinoma.

Fig. 6.—Photomicrograph of fourth generation transplant of tumor from No. 87 CAF_1. ×215.

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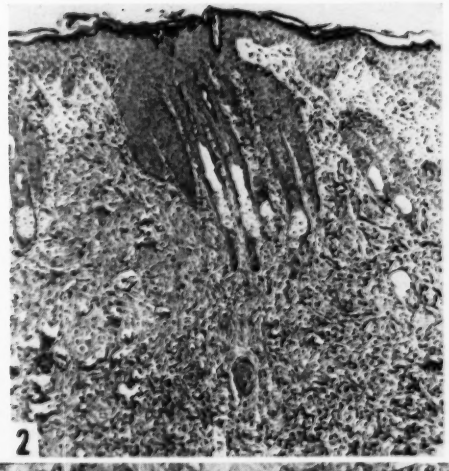

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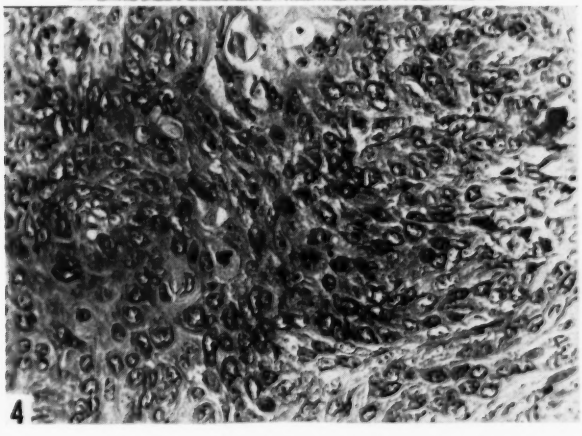

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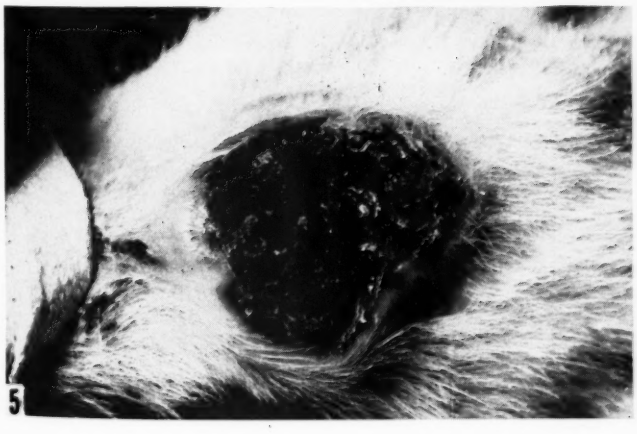

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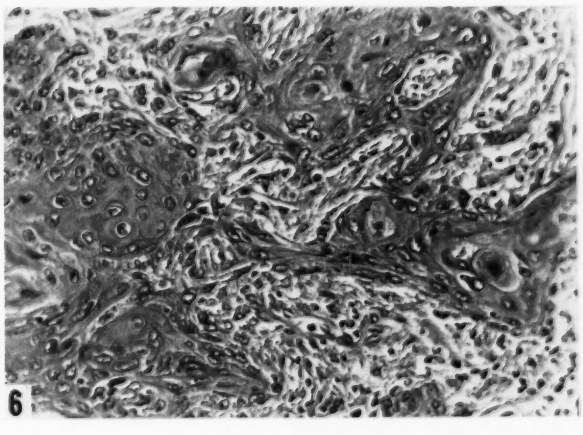

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FIG. 7.—No. 37 CAF_1. Early carcinoma (2 separate lesions), 540 days.

FIG. 8.—No. 37 CAF_1. Advanced carcinoma, 590 days.

FIG. 9.—No. 37 CAF_1. Photomicrograph of lower lesion at 590 days. ×285.

FIG. 10.—No. 16 CAF_1. Early carcinoma, 494 days.

FIG. 11.—No. 16 CAF_1. Advanced carcinoma, 564 days.

FIG. 12.—No. 16 CAF_1. Photomicrograph of carcinoma at 564 days. ×200.

FIG. 13.—No. 15 CAF_1. Early carcinoma, 494 days.

FIG. 14.—No. 15 CAF_1. Advanced carcinoma, 605 days.

FIG. 15.—No. 15 CAF_1. Photomicrograph of carcinoma at 605 days. ×200.

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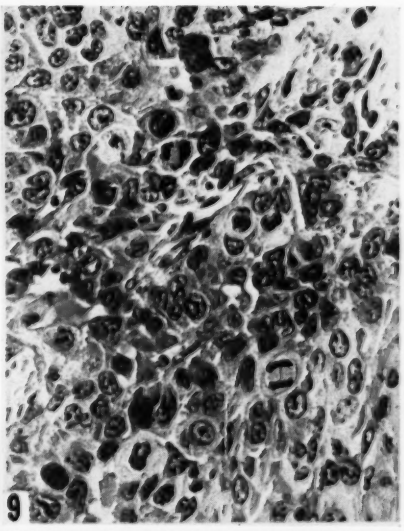
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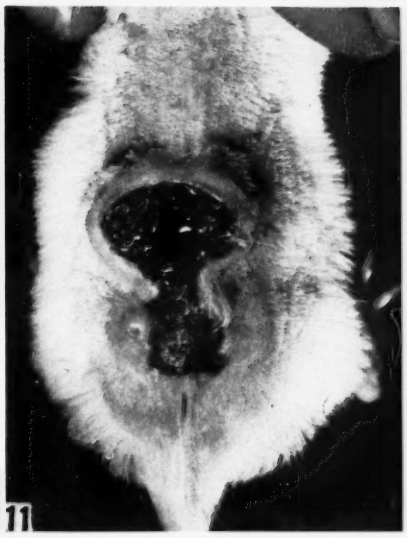
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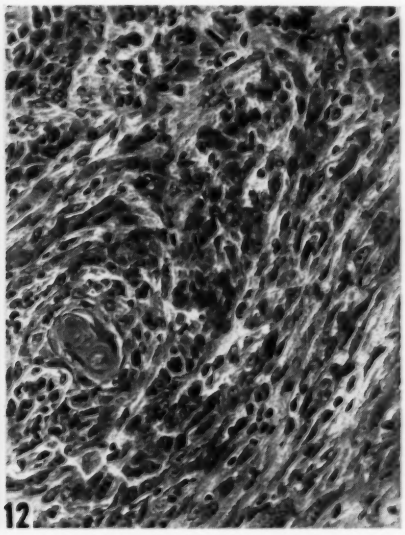
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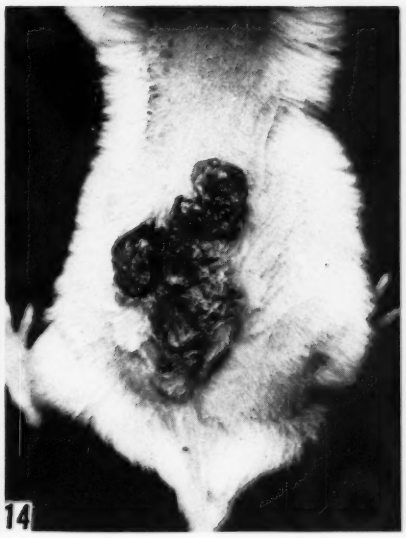
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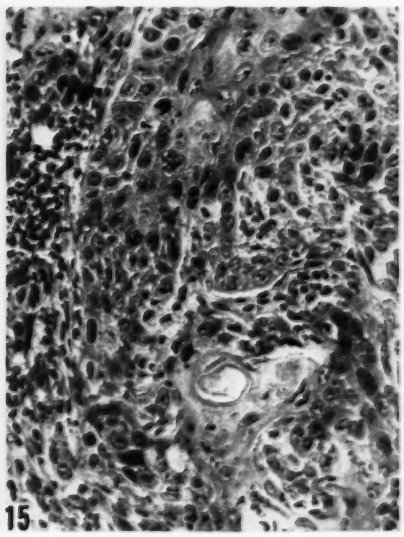
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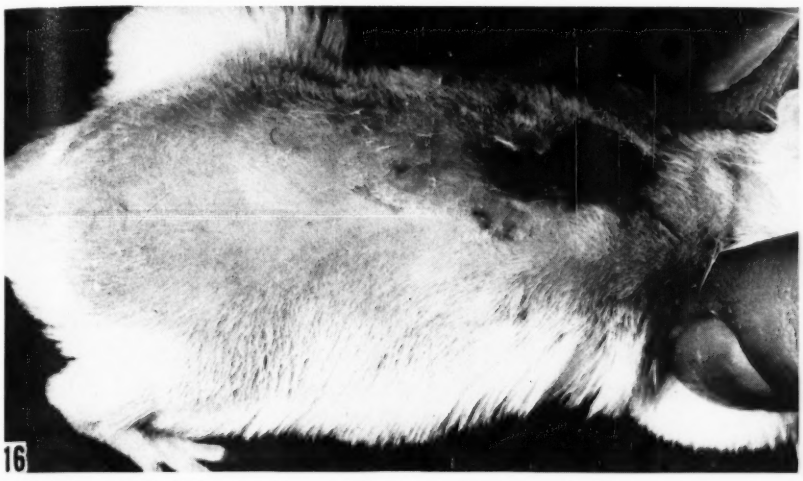
FIG. 16.—No. 3 CAF_1. Beginning carcinoma, 451 days.

FIG. 17.—No. 3 CAF_1. Photomicrograph of carcinoma at 564 days. ×210.

FIG. 18.—No. 3 CAF_1. Advanced carcinoma, 494 days.

FIG. 19.—Photomicrograph of third generation transplant of tumor from No. 3 CAF_1. ×210.

FIG. 20.—No. 3 CAF_1. Advanced carcinoma, 564 days.



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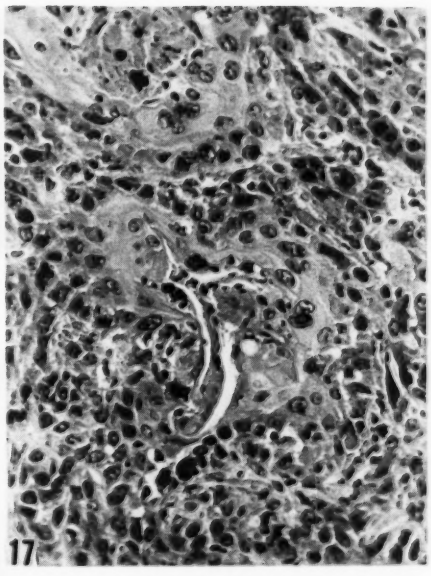

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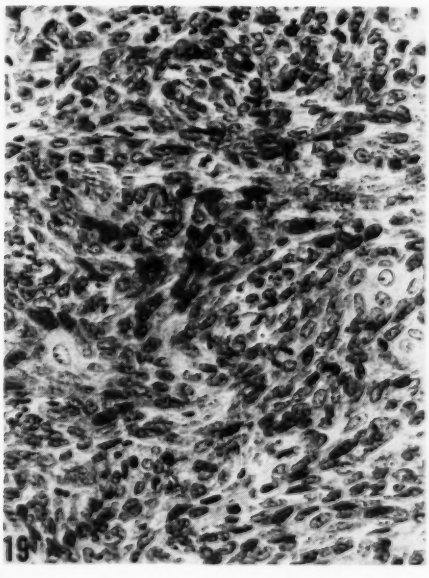

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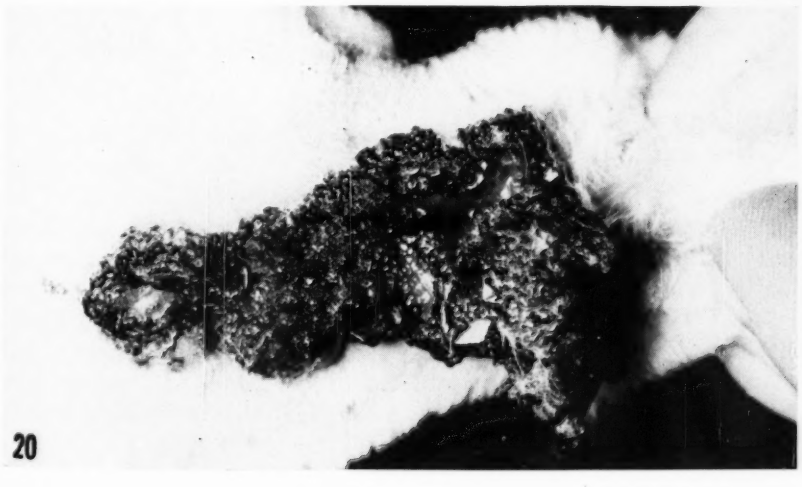

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Synergistic Inhibitory Action of A-Methopterin and a Diaminopyrimidine upon Leukemia L 1210 in Mice*

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INTRODUCTION

Certain 2,4-diaminopyrimidines, synthesized for possible use as metabolic antagonists, were found to have antimalarial activity (3, 5). These pyrimidines were also able to inhibit the growth of *L. casei* and *L. citrovorum*, and this effect could be reversed by folic acid or by folinic acid (4–8). For this reason it has been proposed that these pyrimidines probably inhibited the folic acid-folinic acid conversion (1, 3, 5), as has been proposed for the folic acid analogs (14, 19). This hypothesis has received support from the reports that Sarcoma 180 (2) and leukemia Ak4 (1), tumors inhibited by folic acid analogs, were also inhibited by these pyrimidines.

Data are herewith presented on the effect of one of these drugs, 2,4-diamino-5-(3′,4′-dichlorophenyl)-6-methylpyrimidine (W-50-197)[1] on the survival time and tumor size in mice inoculated with the transplantable leukemia L 1210 (11). These effects are compared to the effects of A-methopterin when given alone and to the effects when the two drugs are given in combination.

MATERIALS AND METHODS

An L 1210 tumor brei, diluted 1:4 with Locke's solution, was inoculated in the axillary spaces of 751 DBA_2 mice. Drug injections, when given, were withheld until 72 hours *after* transplantation. Drugs were administered intraperitoneally every other day for three doses, unless otherwise specified. Aseptic technics were used. Animals were weighed at the beginning of each experiment, once weekly thereafter, and at autopsy. A-methopterin was dissolved in neutral distilled water, while W-50-197 was dissolved in acidulated water.[2] Drugs were diluted so as to deliver the required dosage in mg/kg of body weight in a volume of 0.2 cc. Control mice given inoculations of L 1210 received injections of the diluting fluid alone or were not given injections. Twenty additional mice not inoculated with leukemia L 1210 were used to determine the maximum tolerated dose of W-50-197 for normal DBA_2 mice.

RESULTS

Dose response graphs of W-50-197 (1.25 mg/kg to 20 mg/kg) and of A-methopterin (0.75 mg/kg to 6 mg/kg) appear in Chart 1. One hundred forty-five mice were used in the study on W-50-197 and 122 mice in the study on A-methopterin, with 66 animals used as leukemic controls for both drugs. When W-50-197 was given at a level of 5 mg/kg or less, it was ineffective in increasing the survival time, while doses of 10 mg/kg and 20 mg/kg significantly increased the survival time. A-methopterin was ineffective on this regimen at a level of less than 3 mg/kg but was effective at 3 mg/kg or 6 mg/kg. The higher dose, however, was only slightly more effective in increasing the survival.

The results of combination therapy with the two drugs at effective doses are shown in Table 1. In three series utilizing 99 mice, the combination of A-methopterin (3 mg/kg) and W-50-197 (10 mg/kg) increased the survival time significantly over that of either drug given alone (P less than 0.01).

It was noted, however, that the leukemic animals receiving W-50-197 alone at 10 mg/kg for three doses lost weight. Also, normal animals did not survive the administration of six doses of W-50-197 at this level given every other day. However, normal animals did tolerate without weight loss twelve doses of W-50-197 at 5 mg/kg

* Presented April 7, 1953, at the 38th annual meeting of the American Society of Experimental Pathology, Chicago, Illinois, and published in abstract form (13).

† National Institutes of Health, Public Health Service, Department of Health, Education, and Welfare.

[1] W-50-197 was kindly supplied by the Wellcome Research Laboratories and A-methopterin by the Lederle Laboratories.

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[2] A stock solution of W-50-197 was prepared as follows, as suggested by Dr. George Hitchings of the Wellcome Research Laboratories: 200 mg. were added to 20 cc. distilled water containing 0.1 cc. of glacial acetic acid. The mixture was warmed, cooled, and diluted to 25 cc.

given every other day, and subsequent experiments utilized only the lower dosages of W-50-197 (0.625 to 5 mg/kg).

Further exploration of combination therapy with the two drugs was undertaken in five series of experiments with 214 mice; the drug was given every other day beginning 3 days after transplantation. When A-methopterin (3 mg/kg) was given with graded doses of W-50-197 (0.625–5 mg/kg), the most effective dose of W-50-197 was 2.5 mg/kg. When W-50-197 (2.5 mg/kg) was then given with graded doses of A-methopterin (0.75–3 mg/kg), the most effective dose of A-methopterin was 1.5 mg/kg. From the survival data in the dose response graphs (Chart 1) it can be seen that W-50-197 at 2.5 mg/kg and A-methopterin at 1.5 mg/kg, given singly, are ineffective against leukemia L 1210. However, when the drugs were given in combination at these levels, they exhibited synergism and potentiated the antileukemic action of each other as seen in Table 2. In three series with 105 mice, the combination of A-methopterin at 1.5 mg/kg and W-50-197 at 2.5 mg/kg for six doses increased the duration of survival of leukemic mice from a mean of 8.3 days to a mean of 15.9 days (P less than 0.01).

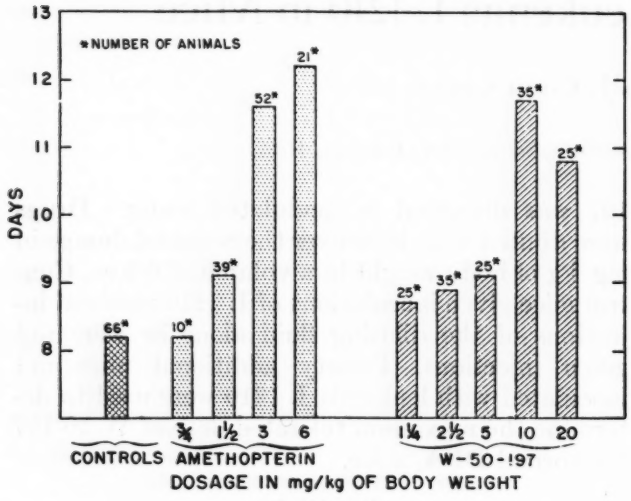

CHART 1.—Survival time of mice receiving A-methopterin or W-50-197 every other day for three doses beginning 72 hours after transplantation of leukemia L 1210.

TABLE 1

COMBINATION THERAPY OF A-METHOPTERIN AND W-50-197 WHEN DRUGS ARE GIVEN AT INDIVIDUALLY *Effective* DOSES (THREE SERIES)

Drug	Dose (mg/kg)	No. mice	Mean survival (days)	Standard error
Untreated		22	8.4	0.38
A-methopterin	3	22	12.3	0.49
W-50-197	10	35	11.7	0.47
A-methopterin	3	20	14.7	0.61
W-50-197	10			

Although W-50-197 was effective in increasing the survival time of mice only at high doses (10-20 mg/kg) when given singly, it was effective in inhibiting local tumor growth even at lower doses. In Table 3 are shown the caliper measurements of the tumor masses in the right axilla 6 days after transplantation and 1 day after the second drug dose was administered. When W-50-197 was given in combination with A-methopterin, retardation of local tumor growth was considerably greater than had been found after therapy with either single drug. However, when a tumor brei, prepared from the spleens of animals with tumors too small to measure, was transplanted into normal mice, the usual manifestations of the growth of leukemia L 1210 appeared within the usual time.

TABLE 2

SYNERGISTIC ACTION OF *Combined* A-METHOPTERIN AND W-50-197 THERAPY WHEN DRUGS ARE GIVEN AT *Individually Ineffective* DOSES (THREE SERIES)

Drug	Dose (mg/kg)	No. mice	Mean survival (days)	Standard error
Untreated		27	8.3	0.31
A-methopterin	1.5	24	9.2	0.64
W-50-197	2.5	25	8.5	0.58
A-methopterin	1.5	29	15.9	1.07
W-50-197	2.5			

TABLE 3

TUMOR VOLUME IN LEUKEMIC ANIMALS ON 6TH DAY AFTER TRANSPLANTATION AND 1 DAY AFTER SECOND TREATMENT

Group	Treatment	Mean tumor volume ($cm.^3$)	Standard error
A	Untreated	2.29	0.46
B	Acetic acid	2.59	0.59
C	A-methopterin*	1.24	0.13
D	Diaminopyrimidine (W-50-197)†	0.74	0.29
E	A-methopterin* and diaminopyrimidine (W-50-197)†	0.10	0.04

* A-methopterin dosage = 1.5 mg/kg.

† 2,4-Diamino-5-(3′,4′-dichlorophenyl)-6-methylpyrimidine dosage = 2.5 mg/kg.

Group C is significantly different from Group E (P less than 0.01) but *not* from Groups A and D.

Group D is significantly different from Group E and from Group A (P less than 0.05) but *not* from Group C.

Group E is significantly different from Groups A and C (P less than 0.01) and from Group D (P less than 0.05).

There was no significant change in weight in any group at the end of the 6th day. Ten animals weighing between 20 and 22 gm. were used in each group.

DISCUSSION

Evidence has been presented that A-methopterin and W-50-197 are more effective as inhibitors of leukemia L 1210 when given in combination

than when given singly. At higher doses the effectiveness of the combination is *less* than additive, while at lower doses the combination is *more* than additive. The synergism at the lower doses is particularly remarkable because it occurs at dose levels at which either drug given alone is ineffective in increasing survival time.

Results of related studies with the diaminopyrimidines, of which W-50-197 is one example, are perplexing and do not adequately delineate the real nature of the mode of action of these drugs. (*a*) In our own experience and in that of others (1, 2), the monochlorophenyl and dichlorophenyl derivatives are both antimalarial drugs and microbial growth inhibitors, but only the dichlorophenyl compounds are tumor inhibitors. (*b*) While the toxic effects of both the monochlorophenyl and the dichlorophenyl compounds, when used as tumor inhibitors, are said to be reversed by folinic acid, in much the same way as the action of the folic acid analogs is reversed by folinic acid (1), the antimalarial activity of a monochlorophenyl derivative was *not* reversed by folinic acid but was reversed by folic acid (4). (*c*) Folic acid antagonists such as A-methopterin inhibit microbial or tumor growth but are not antimalarials. (*d*) Toxicity studies on W-50-63, a monochlorophenyl derivative, indicate that the rat, a species as sensitive as the mouse to A-methopterin, is relatively insensitive to W-50-63, while the monkey is very sensitive (16, 17). (*e*) Studies at the National Cancer Institute on the guinea pig,[3] a species relatively insensitive to A-methopterin (9, 12), indicate that this species is also very sensitive to W-50-197. Such evidence would suggest that the interpretation of the mode of action of these drugs must take into consideration the physiologic response of the species in which they are tested. If A-methopterin and W-50-197 act along the same metabolic pathway as suggested (1, 3), the synergistic action of the two drugs at otherwise subinhibitory single doses supports the viewpoint that the drugs probably act at different points.

It should be emphasized that the injection schedule used in this experiment was weighted against the drugs' effectiveness because of a 3-day lag in the onset of therapy. It must also be pointed out that related toxicity and excretion studies on W-50-63 suggest that the drug regimen of alternate day treatment with W-50-197 at low doses probably prevented the rapid build-up of toxic levels that are manifested in mice given daily doses of the drug (17).

Effective chemotherapy of leukemia has been hampered by the onset of drug resistance (10) which is developed by the host or by the tumor or by both (15). More effective chemotherapy logically should reside in the combined use of drugs (18). Synergism of 6-mercaptopurine[4] and that of 8-azaguanine (10) with A-methopterin are examples of combinations following which more effective chemotherapy has been achieved from the use of drugs with different modes of action. Further benefits of combined therapy can be expected in the utilization of A-methopterin in combination with one or more antimetabolites differing from it in mode of action.

SUMMARY

A-methopterin, a folic acid analog, and 2,4-diamino-5-(3′,4′-dichlorophenyl)-6-methylpyrimidine (W-50-197), an antimalarial drug, were given alone and in combination to test the effect on tumor size and survival of mice with the transmissible leukemia L 1210. Survival data on 751 mice were based on a drug regimen which began 72 hours after subcutaneous transplantation of the tumor with treatment every other day. W-50-197 at 10 mg/kg increased survival time and retarded the local tumor growth, but was toxic. At doses of 5 mg/kg or less there was no increase in survival time, and the local tumor growth was retarded by doses as low as 2.5 mg/kg. A-methopterin at 3 mg/kg increased the survival time and retarded local tumor growth. At 1.5 mg/kg A-methopterin neither increased the survival nor retarded local tumor growth. Combined therapy was more effective than either drug given singly. At high doses the combined effect of A-methopterin and W-50-197 was less than additive, while at low doses the two drugs, given in combination, were synergistic and potentiated each other's antileukemic action more than additively. Thus, combined therapy with A-methopterin at 1.5 mg/kg and W-50-197 at 2.5 mg/kg, doses which were ineffective when given alone, was associated with a significant retardation in local tumor growth and an increase in survival time of 92 per cent.

[3] Guinea pigs did not survive when injected with W-50-197 (10 mg/kg) for three doses given every other day. Death was preceded by leukopenia. At autopsy there was a decrease in the size of the spleen, enlargement of the adrenal glands, severe depletion of the marrow, and, in two of the six animals examined at this dose level, there were nonpenetrating gastric erosions. All six animals contained kidneys in which there was a peculiar lesion of the epithelium of the calyces and anteriad portion of the ureters. This lesion can best be described as a pseudodyskeratosis of the transitional epithelium and probably is similar to the lesion recently described by Schmidt *et al.* (17) in the kidneys of monkeys treated with a related drug, W-50-63 (Daraprim). A report on the effect of diaminopyrimidines in guinea pigs is in preparation (E. M. Nadel).

[4] Dr. H. E. Skipper (personal communication).

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Studies on a Transplantable Chicken Lymphoma (RPL-12)

I. Pathological Changes Following Transplantation of the Tumor*

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Recent observations in this laboratory on the mechanism of virus oncolysis have been largely confined to a study of the effects of virus infections on transplantable tumors (6, 11). The relative importance of the so-called oncolytic agent and the contribution of the host defences in bringing about regression of a transplantable tumor are difficult to assess. In the course of our work with the RPL-12 transplantable lymphoma of chickens, we have confirmed the observations of Olson (10) and Burmester and Prickett (2) that, in serial passage, the inoculation of varying amounts of this tumor could lead to absorption of the inoculum without tumor formation, growth of a palpable tumor with subsequent regression, or growth progressing to the death of the bird. A study of the histological changes during regression of this tumor following infection with an oncolytic virus[1] revealed that phagocytosis of tumor cells plays an important part. It seems desirable, therefore, to assess the nature of the host response to transplantable tumors before attributing regressive changes to virus infection. For this reason, the following experiments were performed.

MATERIALS AND METHODS

Birds.—Except where otherwise stated, 2- to 3-week-old New Hampshire Red chicks were used. All birds were vaccinated against Newcastle disease when 1 day old.

Tumor.—The RPL-12 tumor used in these studies was originally transplanted by Olson (10) from a female cross-bred (Rhode Island Red female × Plymouth Rock male) chicken; it was obtained through the courtesy of Dr. B. R. Burmester of the Regional Poultry Research Laboratory, East Lansing, Michigan, and serially passed in the pectoral muscle. For the preparation of the inoculum, tumors were excised 7 days after transplantation. Pieces of tumor, as free as possible from muscle and necrotic material, were ground up in a Ten Broeck grinder (12) and suspended in physiological saline. Birds were given inoculations in the pectoral muscle of 0.25 ml. of these suspensions, made to various concentrations as indicated in the respective protocols.

Biological procedures.—In most experiments, many of the birds were sacrificed for examination, and the remainder were set aside as controls to assess the ability of the inoculum to produce lethal or spontaneously regressing tumors; the mortality was noted daily for a period of 3 months, and the development of tumors was determined by palpation of the pectoral muscle 3 times a week.

The concentration of tumor tissue in the inoculum, the number of birds in the control and experimental groups, the time of sacrifice, and the purpose of each experiment are shown in Table 1. Twenty control birds which survived implants of heat-killed tumor in Experiment II were subsequently tested for susceptibility to living tumor cells by the injection of a 10 per cent suspension of fresh tumor tissue into the opposite breast 3 weeks later.

To obtain a high rate of regression a small concentration of tumor was used in the inoculum for Experiment IV (Table 1). In Experiment IV-A birds were sacrificed when the tumor showed clinical evidence of regression, with the exception of the first four, which were sacrificed to observe the earlier growth of the tumor. In the second part of the experiment (IV-B), most of the birds were sacrificed when the tumors were large, and before they showed clinical evidence of regression. In Experiment IV-C, birds were sacrificed mainly between the 7th and 17th day after inoculation—the period when the most interesting histological changes were expected.

In Experiment V-A, a group of 73-day-old birds which had survived the inoculation of a 0.01 per cent tumor suspension at the age of 16 days were inoculated in the opposite breast with a 10 per cent suspension of tumor tissue. Experimental and control groups were sacrificed and observed as before (Table 1). In addition, the potency of the second inoculum was tested by the simultaneous inoculation of seventeen birds

* A preliminary report of this work was presented at the 44th Annual Meeting of the American Association for Cancer Research in Chicago, Illinois, April 9–11, 1953, and published in abstract form (7).

[1] R. Love and G. R. Sharpless (to be published).

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TABLE 1

Experimental Data and Summary of Histological Changes at Site of Inoculation

Experiment no.	Purpose To study	Inoculum Per cent tumor suspension	No. birds	Experimental (Birds Sacrificed) — Days after tumor implantation																						Controls (Birds set aside for observations)		
				0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22–190	Total (no.)	Developed tumors (no.)	Died (no.)
I	Early stages of tumor growth	2.0	15	PG 9*†	PG 2	PG 1	PG 1	PG 1	PG 1																		5	5	2
II	Host reaction to heat killed tumor	2.0 (Previously heated to 100° C. for 30 minutes)	14		NG 1		NG 2		NG 2		NG 2			NG 2		NG 2		NG 1			NG 1				NG 1		20	0	0
A		10.0	12		PG 1	PG 1	PG 1	PG 1	PG 1	PG 2	PG 1	PG 1	PG 1	PG 1							PG‡ 1						8	8	7
III B	Course for lethal tumor	1.0	12								PG 2	PG 1	PG 1	PG 1	P 1		PG 2	PG 1				RT 1			RT 1	RT 1	9	9	6
A		0.1	27					PG 1			PG 2				PG 1						RT 2		RT 2		RT 2	RT 17	No Controls		
IV B	Spontaneous regression after first inoculation of tumor	0.1	18				PG 1	PG 1	PG 1		PG 1			PG 1	PG 1	PG 1	P 1	P 2	P 1	P 1	PG 1	P 1	P 1		RT 1	RT 2	30	30	16
C		0.1	29					PG 2		PG 1	PG 2	PG 2	PG 2	PG 3	PG 1 P 1	PG 1 P 1	P 1 RT 2	PG 1 P 1	PG	P	P 1			P‡ 1 P 1	RT 1	No Controls		
A		10.0	13		PG 1		PG 1		PG 1		P 1			RT 2	P 1	RT 1		RT 1			RT 1				RT 1	RT 1	4	0	0
V B	Response to second inoculation of tumor	10.0	13 —	PG 2	PG 2	NG 1 PG 1	PG 1	PG 1	RT 1		RT 1	P 1	RT 1	PG 1												RT 1	6	6	0

NG = No growth of tumor cells.
PG = Stage of progressive growth.
P = Stage of phagocytosis.
RT = Stage of restitution of muscle.
* Numbers indicate number of birds sacrificed at that stage.
† Sacrificed 1, 2, 3, 5, 7, 9, 11, 13, and 15 hours after inoculation.
‡ Found dead.

of the same age, which had not previously received injections of tumor; all developed tumors and died. In preparation for the latter part of this experiment (V-B) a 3 per cent suspension of tumor was inoculated into a group of 16-day-old birds. Regression of the tumor was brought about by treatment with an oncolytic virus 3 days later (11). When 75 days old, these birds were challenged with a 10 per cent suspension of tumor tissue and divided into control and experimental groups as before (Table 1). The potency of the second inoculum was confirmed by the simultaneous inoculation into nine birds of the same age which had not previously received injections of the tumor: all developed tumors, and six died.

The normal histology of birds of comparable age to the experimental groups was determined in twelve chickens sacrificed at the rate of three a week from the age of 3 weeks.

Pathology procedures.—All birds of the experimental groups were killed with chloroform and autopsied. Blocks were taken from both pectoral muscles, skeletal muscle of thigh, sciatic nerve, spleen, femoral bone marrow, thymus, bursa of Fabricius, liver, pancreas, esophagus, proventriculus duodenum, ileum, large intestine, cecum, thyroid, suprarenal, gonad, kidney, heart, larynx, trachea, and lung. For routine purposes, tissues were fixed in formol sublimate, and paraffin sections were stained with hematoxylin and eosin. Where finer detail was required, sections were stained by Barrett's bone marrow method (1). Six to ten blocks of the injected muscle were prepared in the first instance, and, where the tumor could not be detected, serial sections were made.

RESULTS

Controls.—The viability of the inoculum was confirmed by the development of palpable tumors in all controls receiving the first inoculation of tumor. In the experiments designed to produce a fatal outcome (Experiments III-A and B), 87 per cent and 67 per cent of the birds died. In the group in which spontaneous recovery was attempted, 46 per cent of the controls survived (Experiment IV-B, Table 1). Resistance to a second inoculation of tumor was shown by the fact that all birds survived reinoculation with a heavy dose of viable tumor (controls, Experiment V), whereas 23 of 26 birds of the same age died when they were given the same inoculum for the first time. All birds which had previously been given heat-killed tumor developed tumors, and all died after challenge with viable tumor.

When the tumor was lethal, most of the birds died within 14 days and none later than 26 days after inoculation. The birds that died had massive tumors extending the whole length of the sternum with extensive edema of the surrounding tissues and occasional secondary deposits in the proventriculus, pancreas, lung, heart, and gonads.

Many birds of the normal control and of the experimental groups showed signs of mild chronic respiratory disease, but this was equally common in both groups.

Experimental: Histological changes in the muscle following the first inoculation of tumor.—During the first 48 hours after inoculation of the tumor, the perimysial, and to a lesser extent the endomysial spaces of the pectoral muscle, contain linear aggregations of eosinophilic granular material and degenerating muscle fibers mingled with nuclear fragments and scanty primitive cells resembling lymphoblasts (Fig. 1). Since these later come to comprise the vast majority of cells in the tumor (Figs. 2 and 3), they will be designated tumor cells. They are large, round cells 9–15 μ in diameter; each has a prominent nucleus which is more or less centrally placed in the moderately basophilic cytoplasm. The nucleus is round, oval, or slightly indented, and has a well defined nuclear membrane and a large nucleolus (Fig. 3). Occasional tumor cells in mitosis are present as early as 3 hours after implantation.[2]

Around the linear collections of debris and tumor cells there are moderate amounts of hemorrhage, edema, and inflammatory cellular reaction consisting of neutrophil polymorphs, macrophages, and a few small lymphocytes. At first, the inflammatory cells are confined to the vicinity of small veins, but within a few hours after inoculation they mingle with the debris of the inoculum. Throughout this period, increasing numbers of lymphocytes appear around the small veins in the surrounding muscle (Fig. 1).

The possibility of the inflammatory reaction being the result of differentiation of the lymphoblastic tumor cells or of virus activity in the inoculum was excluded by examining sections of muscle after the inoculation of heat-killed tumor tissue. The reaction in this case is identical to that observed with the living tumor cells, except that macrophages are more numerous, and occasional foreign body giant cells are found; no large lymphoblastic cells are present in the necrotic debris (Fig. 4).

Forty-eight hours after inoculation, tumor cells can be observed in the perimysial and endomysial spaces at some distance from the debris of the inoculum which appears to be undergoing phagocytosis by the macrophages. Four days after implantation the nonviable remnants of the inoculum are no longer recognizable, and there is considerable infiltration of the perimysial and endomysial spaces by tumor cells showing numerous mitotic figures. Tumor cells are commonly found in the wall and lumen of veins and in the lymphatics of the muscle.

The tumor continues to grow for the next 6–13 days; it now comprises a large central zone of muscle fibers widely separated by sheets of closely packed tumor cells (Fig. 2) and a periphery where

[2] A study of the cytochemistry of these cells will be presented in a subsequent paper.

scanty perivenous collections of lymphocytes and some edematous fluid containing occasional macrophages mingle with the tumor cells. In the surrounding muscle a few perivenous lymphocytic foci are present as before. At this stage, areas of ischemic necrosis involving tumor cells and other tissues appear in the center of the tumor. The tumor cells appear to undergo two types of degeneration. In the center a form of coagulation necrosis occurs, and all stages can be observed from a general loss of affinity for stain to a shadow cell comprising a structureless eosinophilic mass. Around the edges and in the smaller necrotic areas, tumor cells appear to undergo shrinkage, loss of internal structure in the nucleus, pyknosis, and, finally, karyolysis; in a few cells karyorrhexis precedes the final solution of the chromatin. Cuffs of tumor cells remain around the arterioles (Fig. 5) and, to a much lesser degree, around the dilated, thin-walled veins. The smaller necroses are associated with some inflammatory reaction consisting of neutrophil polymorphs and macrophages; the larger areas of necrosis, however, which appear after the 9th day of growth provoke little or no inflammatory response until the surrounding viable tumor tissue shows evidence of regression.

Throughout the first 10 days, therefore, and in some birds up to the 17th day after inoculation, the tumor continues to infiltrate the muscle and increase in size (stage of progressive growth, Table 1). In some birds between the 11th and 17th days, and in all birds sacrificed or dying between the 17th and 21st days, the tumor shows histological evidence of regression (stage of phagocytosis, Table 1). The onset of regression is characterized by phagocytosis of tumor cells around the veins in the main mass of the tumor, and, more diffusely, at the edge (Figs. 6, 7, and 8). Numerous macrophages appear in the lumen and walls of the veins and in the perivenous areas of the tumor (Fig. 6). Apparently undamaged tumor cells—some even in mitosis—can be observed within macrophages, where they undergo intracellular digestion. Irregular shrinkage of the nucleus and cytoplasm occurs, and there is margination of the chromatin and swelling of the nucleolus (Figs. 9 and 10). Occasionally, an extreme form of irregular nuclear shrinkage results in the production of pseudopodial projections which are nipped off to form micronuclei (Fig. 10). The process of intracellular degeneration is completed by karyolysis, loss of cytoplasmic basophilia, and solution of the cell.

The onset of phagocytosis is rapidly followed by a great increase in the perivenous lymphocytic reaction in and around the tumor (Fig. 11). The last cells to undergo phagocytosis are the mantles of cells which survive around the anterioles in the areas of central necrosis. By the time these cells are affected, there is extensive diffuse inflammatory cellular infiltration of the widely separated muscle fibers which were previously the site of the tumor. In addition to macrophages and increasing numbers of lymphocytes, large numbers of plasma cells appear in the exudate.

After the 20th day in all birds, and occasionally before this, tumor cells are no longer present, and the process of restitution of the muscle begins (stage of restitution of muscle, Table 1). Numerous lymphocytes and lymphoblasts accumulate around the veins, and germinal centers develop. Syncytial masses of sarcoblasts, new capillaries, macrophages, and scanty fibroblasts invade the necrotic areas. Meanwhile, the diffuse inflammatory cellular reaction in the rest of the muscle diminishes. Finally, the inflammatory exudate is absorbed, the muscle fibres are largely restored, and the lymphoid hyperplasia subsides. A month to 6 weeks after inoculation, the only abnormality in the pectoral muscle consists of a few foci of lymphocytes (Fig. 16) and occasional minute areas of fibrosis.

Histological changes in other organs and tissues.—The only important abnormalities in the other organs and tissues are as follows: (*a*) direct extension of the tumor into the neck and through the skin, (*b*) numerous tumor cells in the capillaries of the lung, liver, or spleen, (*c*) secondary growths in the proventriculus, pancreas, lung, heart, and gonads, and (*d*) changes in the spleen.

Direct extension into the neck is common, and ulceration of the skin is produced by the largest tumors. In three out of 113 birds the capillaries of the lung, liver, and spleen are packed with tumor cells. Secondaries occur only in birds sacrificed, or dying, between the 10th and 20th days after implantation of the tumor, and of 43 birds in this category approximately one-quarter have histological evidence of secondary growth. In all birds in which the primary tumor is regressing, similar changes, including extensive phagocytosis of tumor cells and lymphoid hyperplasia, are present in the secondary deposits.

The changes in the spleen are of extreme interest. The normal chicken spleen (Fig. 12) is essentially similar to that of mammals. The white pulp is arranged eccentrically around the arterioles, and occasional well defined lymph follicles are present. The sheathed arteries are surrounded by three or four layers of concentrically arranged reticulum cells and a small outer zone of mononuclears. The red pulp contains a high proportion of lymphocytes, occasional lymphoblasts, plasmablasts,

plasma cells, mast cells, and the formed elements of the peripheral blood.

During the stage of progressive growth of the tumor, there is an increase in the number of plasmablasts and plasma cells in the pulp, especially around the sheathed arteries (Fig. 13). Towards the end of this phase this is accompanied by a hypertrophy and hyperplasia of the reticulum cells of the sheathed arteries (Fig. 14). Some of the more peripheral reticulum cells become rounded off and develop hyperchromatic nuclei with a prominent nucleolus and deeply basophilic cytoplasm. All gradations from these cells to mature plasma cells can be observed.

With the onset of phagocytosis in the tumor, there is congestion of the pulp and sinuses and deletion of the lymphoid tissue. The peripheral reticulum cells and mononuclears of the sheathed arteries are separated by blood channels and incorporated into the red pulp. Both pulp and sinuses now contain large numbers of mononuclear cells. Meanwhile, the plasma cells continue to increase in the red pulp.

By the time all the tumor cells have disappeared and large numbers of lymphoid and plasmacytic cells are present in the muscle, the sheathed arteries are reduced to normal or subnormal proportions, lymphocytes reappear in considerable numbers, the white pulp increases in size, and prominent germinal centers develop (Fig. 15). The plasmacytic cells in the spleen steadily diminish in number, and finally the lymphoid hyperplasia subsides. Four to 6 weeks after inoculation, when the pectoral muscle is restored and only a few lymphoid foci remain at the site of the tumor, the spleen is no longer distinguishable from that of a normal bird of the same age.

There are considerable variations in the amount and character of the lymphoid tissue in the rest of the body of the normal and experimental birds, but it is not possible to detect any consistent alterations comparable to those observed in the spleen.

The spleens of birds inoculated with heat-killed tumor do not show these changes and, indeed, show no significant abnormality.

Histological changes following the second inoculation of tumor into birds surviving the first implantation.—In the pectoral muscle the stages of progressive growth, phagocytosis of tumor cells, and restitution of muscle are essentially the same as after the first inoculation of tumor. The tumors, however, are much smaller, and the onset of phagocytosis is earlier (Experiment V, Table 1). Tumor cells are not found in the pectoral muscle later than the 11th day after the second inoculation. Secondary growths do not occur.

TABLE 2

SUMMARY OF PATHOLOGICAL CHANGES FOLLOWING TRANSPLANTATION OF THE TUMOR

Duration (days)	Tumor cells	Host reaction	
		Local	Distant-spleen
4	Progressive growth	Phagocytosis of non-neoplastic debris of inoculum. Some lymphocytic reaction.	No changes
6–13		Minimal macrophage and lymphocytic reaction at periphery.	Increased plasma cells. Hypertrophy of sheathed arteries.
3– 4	Phagocytosis and intracellular digestion	Extensive macrophage lymphocyte and plasma cell infiltration.	Great increase in plasma cells. Depletion of sheathed arteries. Diminution of lymphocytes.
7–14	Absent	Increasing lymphocyte and plasma cell infiltration. Absorption of macrophages. Muscle repair.	Decreasing plasma cells. Lymphoid hyperplasia.
7–14	Absent	Diminishing lymphocytes and plasma cells.	Return of lymphoid tissue and plasma cells to normal.

Plasmacytic and lymphoid changes in the spleen follow the same pattern in relation to the muscle pathology. The plasma cell increase is less striking, begins earlier, reaches a peak in the late stage of phagocytosis, and is no longer detectable 3 weeks after inoculation. Definite reticulum-cell hyperplasia was present in two birds only, on the 8th and 10th days after challenge. Lymphoid hyperplasia of moderate degree is limited to the early stages of restitution of the muscle.

DISCUSSION

The first inoculation of tumor results in two main processes: growth of tumor cells and host reaction, which are summarized in Table 2. Despite the presence of occasional macrophages the tumor cells are not susceptible to phagocytosis and produce minimal local reaction during the first 10 days after inoculation. Between the 10th and 21st days a change occurs in the tumor cells whereby they become susceptible to phagocytosis and provoke an extensive local inflammatory response. The phenomenon is essentially similar to the opsonin effect of antibodies on bacteria.

The host has overcome invasion by tumor cells.

Furthermore, regression of the neoplasm is followed by a state of increased resistance to a second inoculation of tumor in a *different site.* Such a phenomenon is strongly suggestive of the presence of circulating antibody.

The association of plasma cells, lymphocytes, and reticulum cells with the production of antibodies is well known. In a recent review of this subject Delaunay (3) implicates all three types of cells, although other workers (4, 5, 9) favor the plasma cell in particular. Makinodan *et al.* (8) recently described plasmacytic hyperplasia in the spleens of chickens developing precipitins to bovine serum albumen. Ehrich and his co-workers (4) observed a plasmacytic, followed by a lymphocytic, hyperplasia in the lymph nodes of rabbits during the development of antibodies to typhoid vaccine. Since the lymphocytic reaction was maximal after the antibody formation had passed its peak, they concluded that the plasma cell and not the lymphocyte was responsible for antibody production.

Throughout the stage of progressive growth of this tumor there is a hyperplasia of the plasma cell series and later of their precursors, the reticulum cells of the sheathed arteries in the spleen. Just before the plasma cells attain their maximum number in the spleen, phagocytosis of tumor cells occurs. Such changes do not occur in birds inoculated with heat-killed tumor which are not resistant to subsequent challenge with tumor. It would seem reasonable, therefore, to suggest that the plasma cells contribute to the production of antibodies.

The role of the lymphoid tissue is more difficult to assess. There are no changes in the splenic lymphoid tissue during the stage of progressive growth of the tumor, and the small amount of lymphocytic reaction in the muscle remains constant throughout this phase and does not seem to interfere with the proliferation of the tumor cells. A similar local reaction occurs after the inoculation of heat-killed tumor, which does not produce immunity. Phagocytosis of tumor cells begins *before* the onset of extensive local lymphocytic infiltration. The transient depletion of lymphocytes in the spleen at this time may be the result of lysis of lymphocytes with liberation of antibody, or may represent the mobilization of lymphocytes to the muscle. If the former explanation is true, the final stage of local and splenic lymphoid hyperplasia could be a compensatory replacement phenomenon.

Reticulum-cell hyperplasia in the spleen occurs in the later stages of progressive growth of the tumor and undoubtedly contributes to the formation of plasmablasts. The majority of the reticulum cells, however, become rounded off towards the periphery of the sheathed arteries and form monocytes or macrophages. Hence, the onset of macrophage infiltration of the tumor coincides with a sudden depletion of the reticulum cells and mononuclears in the sheathed arteries of the spleen.

SUMMARY

1. Inoculation of varying amounts of the RPL-12 lymphoma can lead to growth of a palpable tumor with subsequent regression or to growth progressing to the death of the bird.

2. Regression of the tumor is followed by resistance to subsequent challenge with the same tumor, which is greater than that of normal birds of the same age.

3. Two phases are recognizable in the life cycle

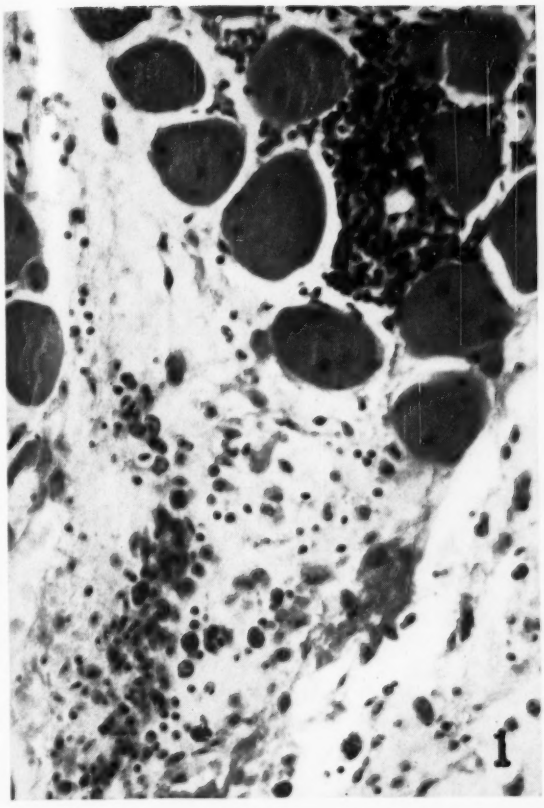
FIG. 1.—Pectoral muscle 26 hours after inoculation of tumor. Note large lymphoblastic cells in inflammatory exudate, and perivenous lymphocytic reaction in adjacent muscle. H and E (Hematoxylin and Eosin) stain. ×300.

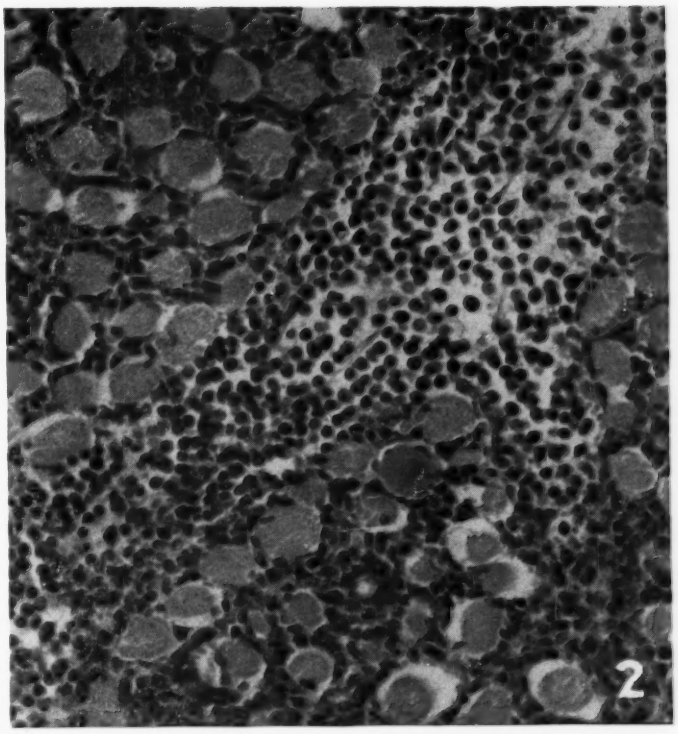
FIG. 2.—Extensive infiltration of pectoral muscle by tumor cells 8 days after inoculation of tumor. H and E stain. ×300.

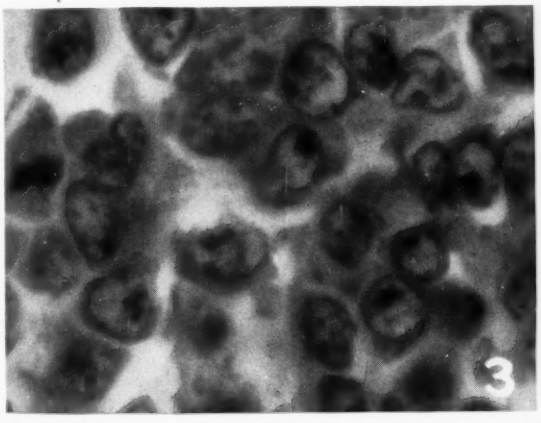
FIG. 3.—Higher magnification of tumor cells. H and E stain. ×1,620.

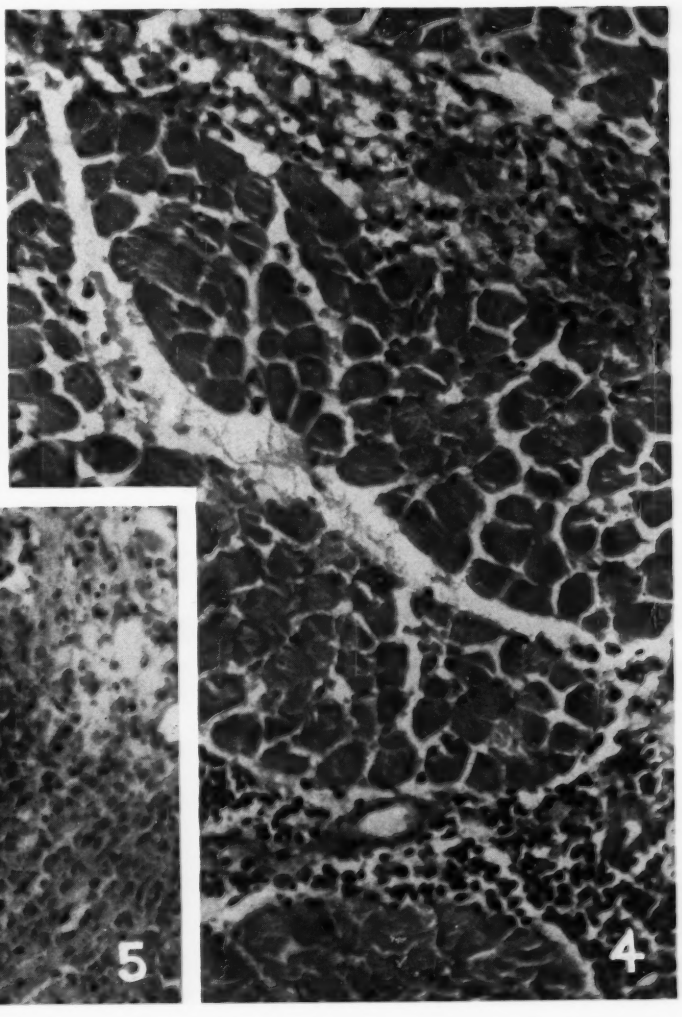
FIG. 4.—Pectoral muscle 24 hours after inoculation of heat-killed tumor cells. Note inflammatory exudate above, and perivenous lymphocytic reaction below. H and E stain. ×300.

FIG. 5.—Mantle of tumor cells around an arteriole in an area of ischemic necrosis. H and E stain. ×300.









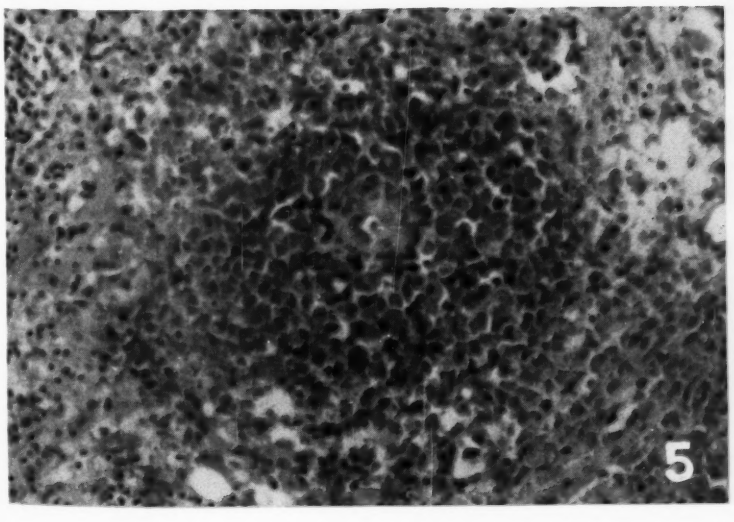


FIG. 6.—Macrophage infiltration around a vein in the center of the tumor. Note intact tumor cells at top and bottom. Barrett's stain. ×760.

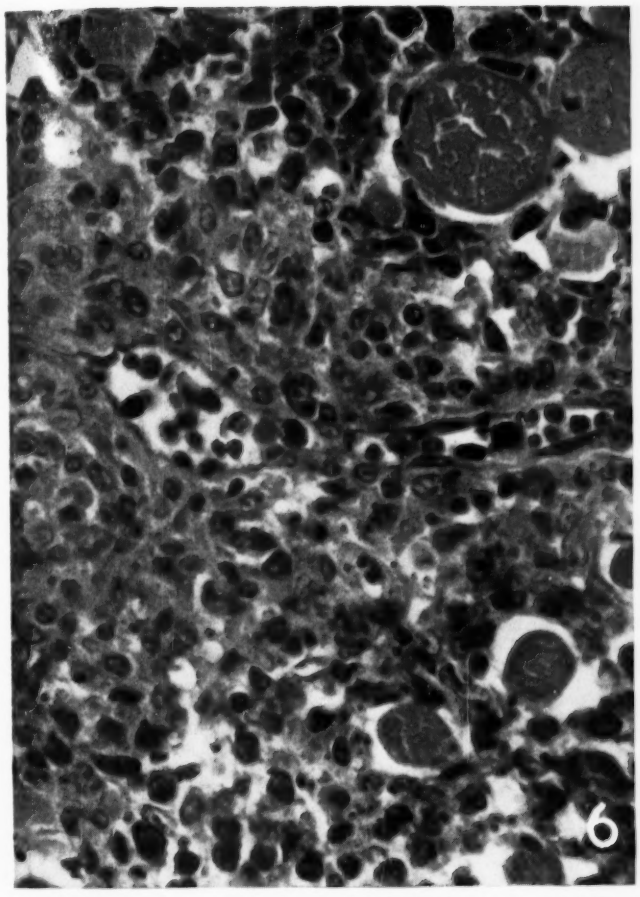
FIG. 7.—Several macrophages containing tumor cells near edge of tumor. Barrett's stain. ×950.

FIG. 8.—Degenerating tumor cells within macrophages, and apparently undamaged tumor cells nearby. Barrett's stain. ×1,620.

FIG. 9.—Nucleolar swelling and margination of chromatin of tumor cell inside a macrophage. H and E stain. ×1620.

FIG. 10.—Main nucleus and a small micronucleus formed by a tumor cell undergoing intracellular degeneration. H and E stain. ×1620.

FIG. 11.—Perivenous (*top*) and diffuse infiltration of regenerating muscle by lymphocytes and plasma cells, after phagocytosis of tumor cells. H and E stain. ×300.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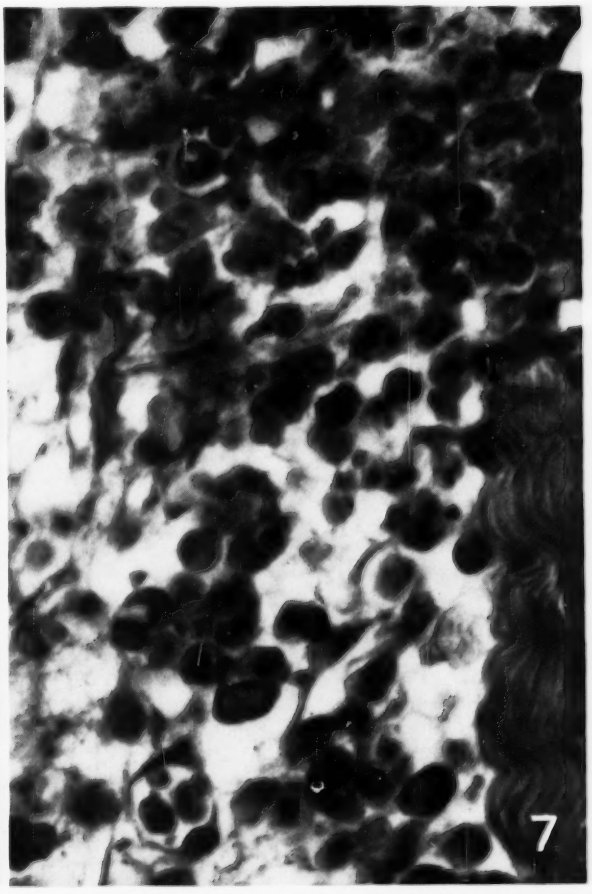
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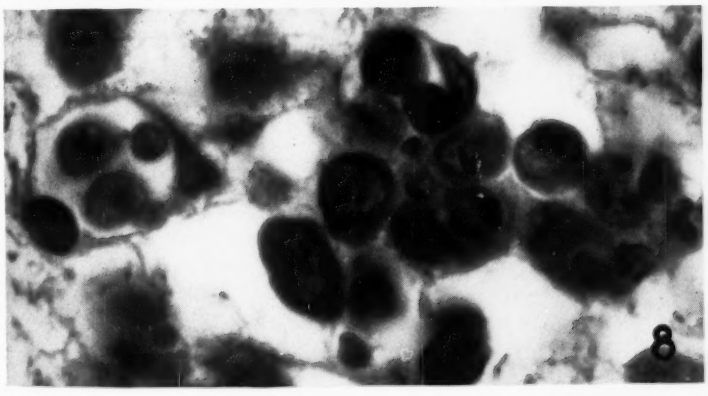
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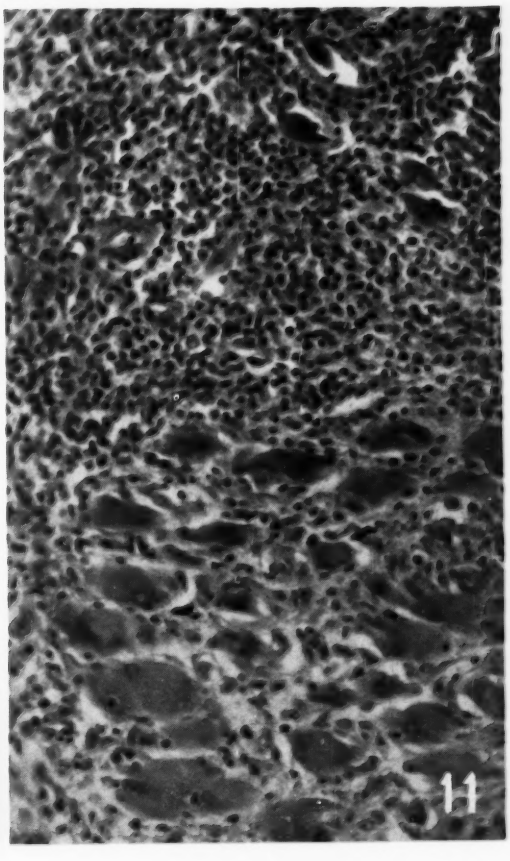
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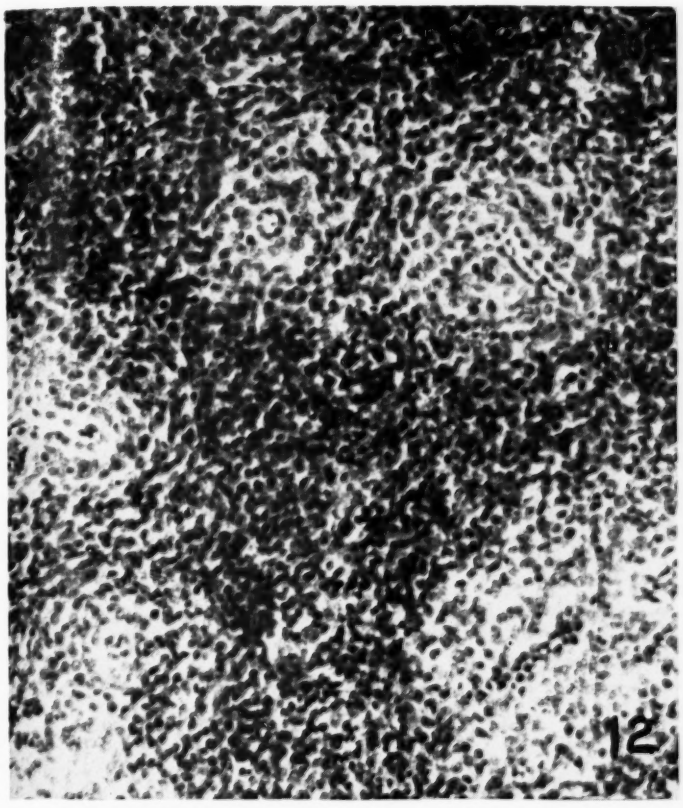
FIG. 12.—Normal chicken spleen showing pale staining reticulum cells and mononuclears of sheathed arteries, and numerous lymphocytes in the red pulp. H and E stain. ×300.

FIG. 13.—Large numbers of plasmablasts and plasma cells in splenic pulp during late stage of progressive growth of tumor. H and E stain. ×1620.

FIG. 14.—Spleen during stage of phagocytosis of tumor cells in the muscle. Note greatly enlarged sheathed arteries and depletion of lymphocytes in pulp. H and E stain. ×300.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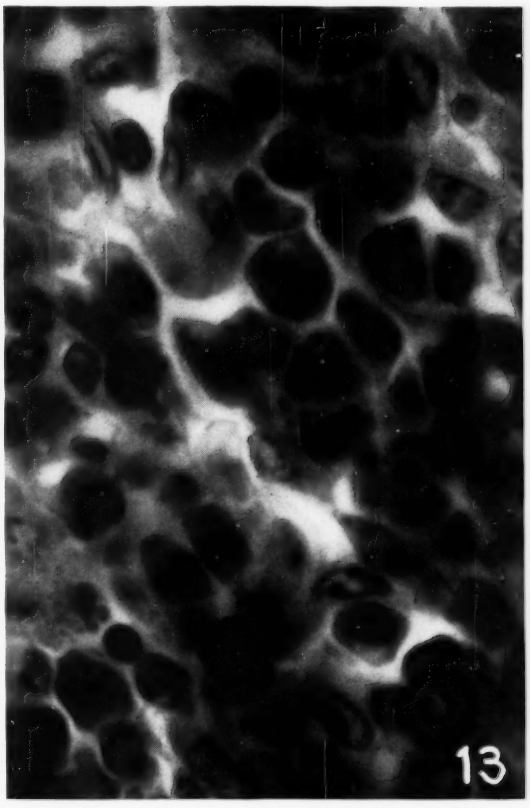
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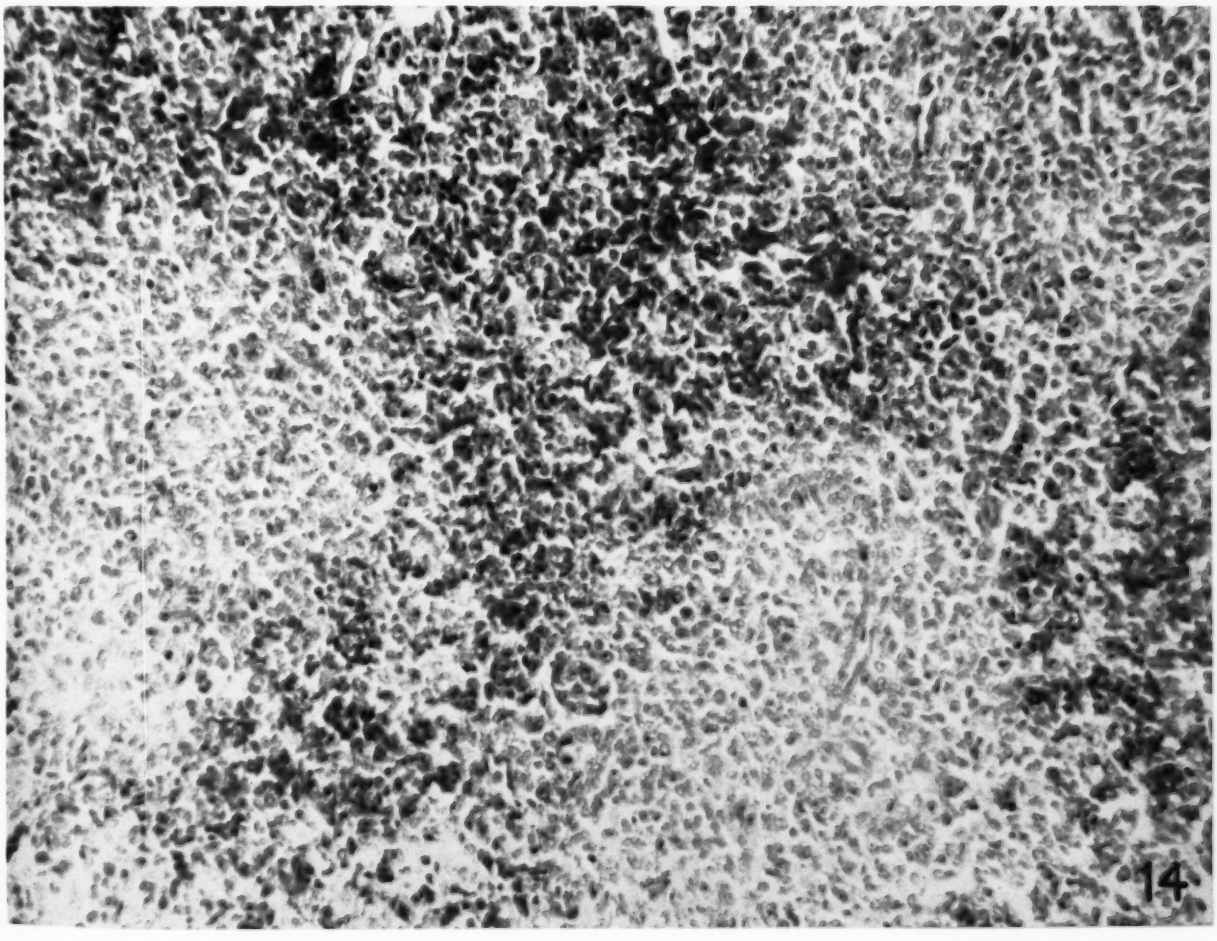
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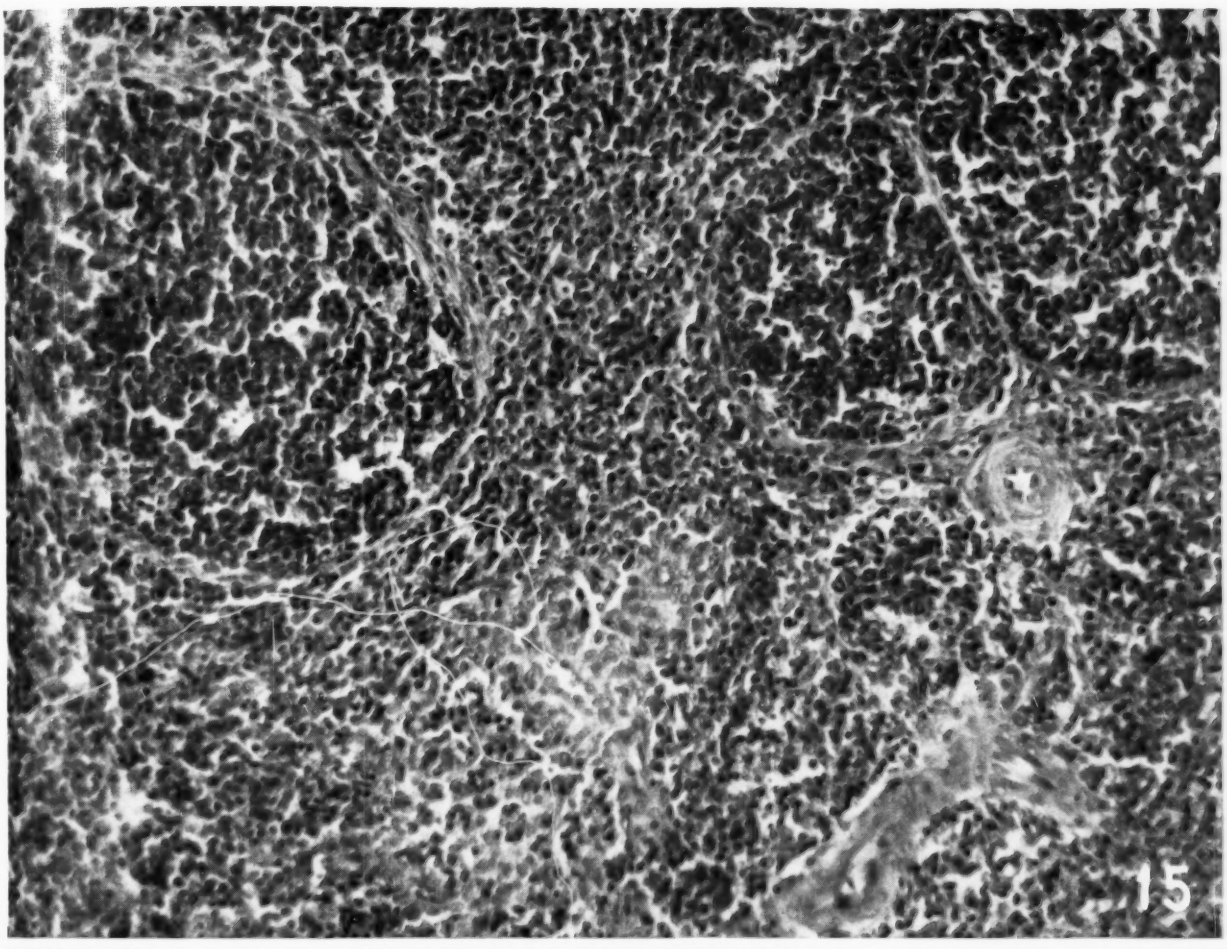
FIG. 15.—Conspicuous germinal centers and small sheathed arteries in spleen during early stage of restitution in muscle. H and E stain. ×300.

FIG. 16.—Small lymphoid focus in pectoral muscle 6 weeks after inoculation of tumor. H and E stain. ×150.



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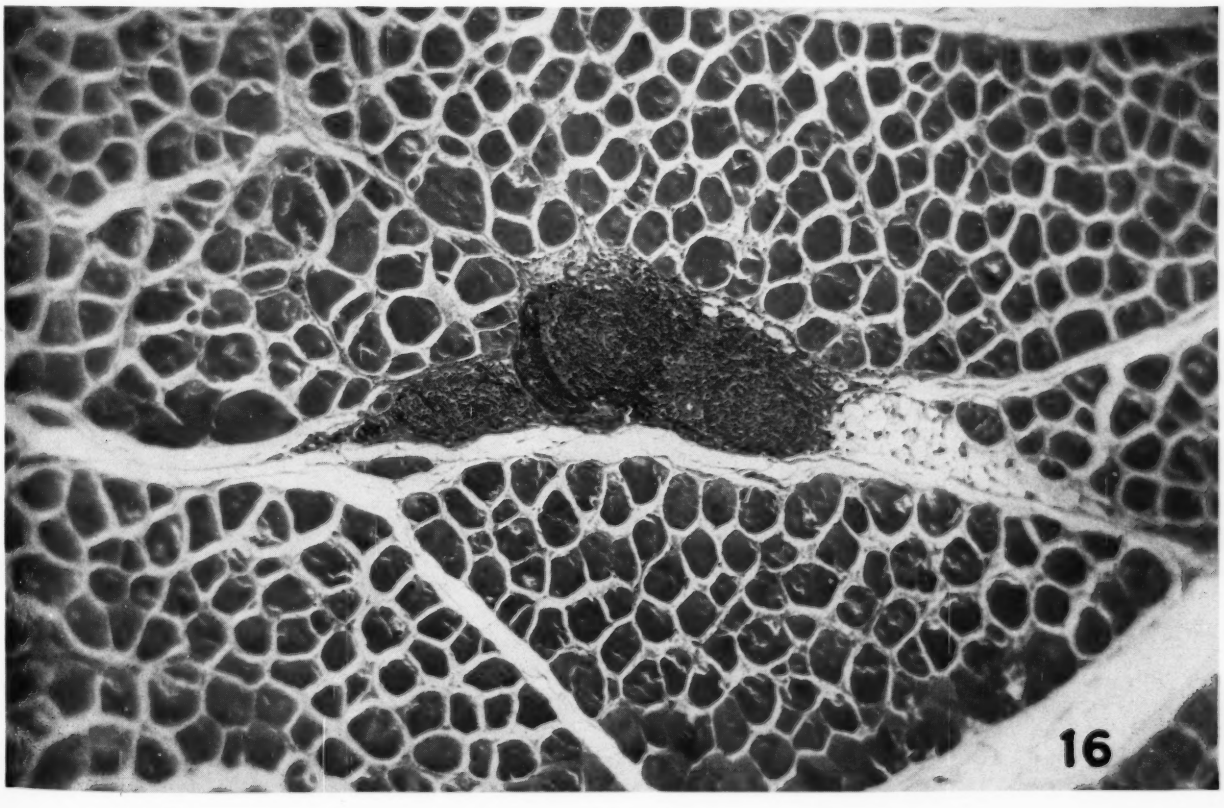

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of the implanted tumor cells. The first, a stage of progressive growth, is of 10–17 days' duration. The second, a stage of regression, is characterized by phagocytosis of apparently intact tumor cells, and is of 3–4 days' duration.

4. The nature of the local and distant host reaction to the implanted tumor is described, and the changes which occur in the lymphoid and plasma cell series are interpreted as the morphological manifestations of an immune reaction.

5. Inoculation of heat-killed tumor tissue is not followed by the changes in lymphoid and plasma cells which accompany a progressively growing tumor and does not give rise to any resistance to subsequent challenge with live tumor cells.

ACKNOWLEDGMENTS

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An Increase in Host Specificity of a Transplantable Spindle-Cell Tumor in Mice*

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A tumor of the forestomach of a mouse of the pBr inbred strain which had been treated with methylcholanthrene has been used in these experiments. Bagshaw and Strong (1) reported the origin of this tumor and previous transplantation experiments with it. It may be referred to as the "pBr spindle-cell tumor," since it arose in the pBr (pink-eyed brown nonagouti) inbred strain of this laboratory and because it is typically made up of spindle-shaped cells with large vesicular nuclei. The individual cells vary greatly in size in sections and interlace with one another at various angles. The histopathology of this tumor has been discussed (1), and for reasons presented there the descriptive term spindle-cell tumor is used.

The tumor has been transplanted subcutaneously in mice of the pBr strain at 5–8 weeks of age, with very uniform results. All the present transplants were made by the author, routinely. The tumor tissue is relatively soft and may fragment while being passed through the trocar.

Beginning with the 36th transplant generation (T_{36}), weights of the tumor were taken at 14 days. By this time the tumor typically forms an elongated hollow mass, and the center is generally filled with blood and tissue debris. Many of the tumors present a segmented or beaded appearance, possibly from fragmentation of the tumor. Weights do not include fluid or tissue debris; in pBr mice the tumors at 14 days average between 0.8 and 1.0 gm., quite consistently. No regressions of tumors have appeared in mice of the pBr strain.

Also beginning at the T_{36} generation, transplants were made into mice of the N strain, one of the three strains in the ancestry of the pBr strain. Surprisingly, the tumor grew, though much more slowly than in pBr mice (Chart 1). It has been carried for 26 transplant generations in the N strain mice, with weights being made on the 14th day. Though consistently slow, it has shown a good deal of irregularity of growth. The tumor grew in 60.5 per cent of mice of the N strain between T_1 and T_9 and in 100 per cent of all mice between T_{10} and T_{26}.

In the ninth transplant generation in N mice, the tumor was returned to mice of the pBr strain, and this series will be referred to as the "N-adapted" transplant. Therefore, we are dealing with three transplant series in all: the original in pBr mice; the series in N mice; and the "N-adapted" tumor in pBr mice.

The N-adapted transplants regained the ordinary growth rate in pBr mice rather slowly (three transplant generations) but then proceeded to grow faster than usual, reaching a peak, six transplantations after return to pBr, at 1.4 gm. average weight, compared to 0.8 gm. for the original tumor growing in pBr mice. Thereafter the growth rate fell back to the level of the original series, though it has not been so steady. In the last five generations it has been considerably slower than the original, though still not so slow as the N series.

Additional information has also been gained in tests employing hybrids between mice of the N and pBr strains. The tumors, both the original and the N-adapted one, had grown in all mice of the N strain at the time the crosses were made between mice of the N and the pBr strains (see Table 1). Tumors of the T_{49}–T_{60} (original series) were employed in these tests, as well as N-adapted tumors between the thirteenth and seventeenth transfer generations (following return to pBr hosts). The data are summarized in Table 1. Weights of these tumors in hybrid mice were not determined, as the mice were allowed to live until killed by the tumors. The present analysis is based on the percentage susceptibility in the various generations rather than the growth rate of the tumors.

It is notable that the tumor grew progressively and rapidly in the F_1 mice—100 per cent. Summarization of all transplant results with pure pa-

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rental types shows 1.4 per cent negatives in pBr (the original series gave these exclusively). The N series gave negatives in the early series T_1–T_9 but no negatives between T_{10} and T_{26} or at the time the genetic crosses were made.

In the F_2 generation, the original tumor grew very well, in general. There was a total of about 5 per cent negatives (see above); there were only 3.3 per cent negatives at 14 days, and the others regressed in the next 2 weeks. However, the N-adapted tumor showed an unexpected number of negatives—at the 14th day 27 per cent of the mice had no tumors, and, of the positives, in 2 or more weeks many more regressed, so that the final percentage of negatives is 45 per cent.

DISCUSSION

The poorer growth of the tumor in the N series must be attributed to genetic differences between N and pBr. These differences are not limited to color genes, in all probability. In the F_2 from the cross, various new combinations presumably occur, some of which are possibly unfavorable for growth of the tumor.

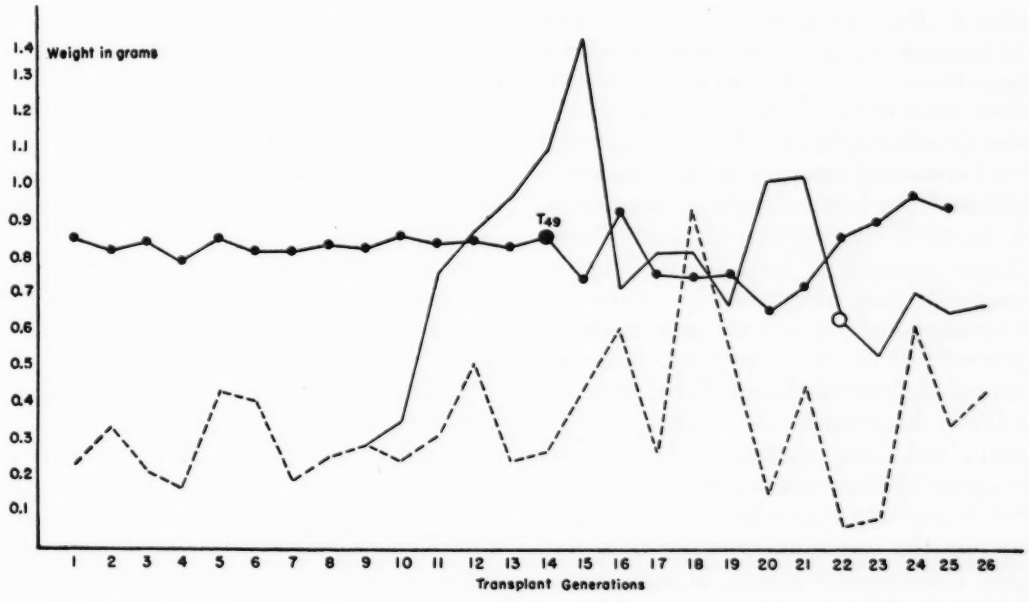

CHART 1.—Data on the average (for either eight or sixteen mice) size of the original spindle-cell neoplasm at 14 days of growth in (*a*) mice of the N-strain (*dash line*), (*b*) mice of the pBr strain (*solid circle, and solid line*), and (*c*) the N-adapted altered tumor in mice of the pBr strain (*solid line*). The transplant generations for mice of the N strain are given on the base line and average weight of tumors along the vertical line. Transfer generations for pBr mice with the original tumor begin with T_{36} (i.e., the 25th generation is really T_{61}).

TABLE 1

THE NUMBERS OF MICE POSITIVE AND NEGATIVE TO THE GROWTH OF THE ORIGINAL SPINDLE-CELL NEOPLASM AND THE ALTERED N-ADAPTED TUMOR DERIVED FROM IT

	pBr	N	F_1*	No. mice	F_2* At 14 days	F_2* Final Classification (30+days)	Per cent positive
Original tumor	489+:7−†	375+:41−	78+:0−	182	176+:6−	163+:19−	94.8±1.6
T_1–T_9		63+:41−					
T_{10}–T_{26}		314+:0−					
Expected ratios (1 gene)						15+:1− =170.6+:11.8−	
"N-adapted"	272+:0−	189+:0−	109+:0−	206	151+:55−	113+:93−	54.9±3.46
Expected ratios (2 genes)					3+:1− =154.5+:51.5−	9+:7− =115.9:90−	
Totals	761+:7−	564+:41−	187+:0−	388			

* From a cross between pBr and N mice.

† + means progressive growth of tumor; − means no growth or only a temporary nodule which completely regressed.

But such facts cannot account for the wide difference of growth success of the original versus the N-adapted tumor in the F_2 mice. It seems more plausible to assume a change induced in the tumor's growth capacities, perhaps in its genetic constitution, as a result of its sojourn in N mice. The difference in the two F_2 ratios, upon which the following discussion is based, as measured by χ^2, is 56.39, a highly significant difference. That the change took place in the N-adapted tumor and not in the original pBr tumor is indicated by the fact that the pBr tumor continues to grow in about 95 per cent of all F_2 mice (unpublished data).

Alterations in the transplantability potentials of tumors in animals are one of the oldest observations in cancer research (20). It was not until 1926, however, that some of these changes were placed on a factorial or genetic basis (18, 19). Since that time, sudden permanent changes in the transplantation of tumors have been verified by several investigators, particularly Bittner (3) and Cloudman (4). These changes in transplantability occurred sporadically but progressively during the continuous transplantation of the tumor over a period of years. The fluctuations were, however, somewhat more than due to chance alone, since the changes, as far as discoverable, were always in the same direction. The tumor progressively acquired the ability to grow in more and more kinds of mice.

Since 1926, many ways have been found to permit a highly specific tumor to grow in more and more animals. These changes have, in part, conditioned the host to tolerate a transplantable tumor, and, in part, probably brought about changes in the tumor cells themselves. It is not always clear, however, whether these changes are of a permanent or only of a temporary nature.

Another problem bearing upon the biological nature of these adapted tumors has also not been convincingly settled. The advocates of the somatic mutation hypothesis for the origin of tumors would like to look upon these altered transplantability potentials as being brought about by some such a process as somatic mutation. However, it should be kept clearly in mind that many tumors, perhaps all, are made up of a mixed population of cells. This is indicated (*a*) by the great pleomorphism of some tumors, (*b*) by the variety of tumors that can be extracted with very little effort from one tumor mass or single cell by tissue culture (10), or (*c*) by transplantation into other animals with only the criterion of a different growth rate in a given group.

There are at least eight ways by which tumor-host relationships can be altered, thus permitting tumors derived from one strain of experimental animal to grow progressively when transplanted into individuals of other strains or even species. These are, as follows:

1. X-raying mice before the transplantation of the tumor (Murphy [14]).
2. Mixing a tumor with cotton or wool fiber before transplantation (Jones [11]).
3. Growing a tumor in the anterior chamber of the eye or in the brain (Green [6, 7], Duran-Reynals).
4. Growing a tumor in an F_1 mouse (Barrett and Deringer [2], verified by Hauschka [8, 9]). This observation is based partly on the fact that susceptibility to transplanted tumors is dominant in crossing tests.
5. The aging of tumors through numerous transplant generations permits successive changes to take place; that is, adapted changes take place spontaneously (Strong [18, 19]).
6. The treatment of the host by estrogenic hormones before the injection of the tumor (Gardner [5]).
7. The treatment of the host by tumor or normal tissue extracts before the inoculation of the tumor (Snell *et al.* [15, 16] and Kaliss and Snell [12]).
8. The growth of tumors in the newborn (Strong [17], Little [13], Gross).

There appear to be different mechanisms involved in these different adaptive procedures. Some changes (X-ray) appear to be altering the host mechanism, whereas other procedures (3, 4, and 5) appear to be bringing about changes in the tumor cells or in tumor cell relationships. (For an excellent review see Hauschka [9].)

The present data demonstrate that the process of "adaptation" may reverse the process of decrease in specificity of transplanted tumors that takes place spontaneously. A dependent tumor which will grow in an F_2 mouse when either one of two alternative genes or factors are present in the genetic constitution of the host (indicated by a 15+ : 1− ratio in the F_2) has now become dependent for its progressive growth on the simultaneous presence of two genes (9+ : 7− ratio in F_2). What relation, if any, exists between the two alternative genes in the growth of the original tumor and the two dependent genes for the survival of the N-adapted or derived tumor has not, as yet, been determined.

It is also not clear whether this increased dependence of the derived tumor has occurred spontaneously or has been brought about through adaptation. One can only select between these two alternative explanations by repeating the adaptation of the pBr or other strain tumors into mice of the N or other strains repeatedly.

Selection between the two ideas whether (*a*)

somatic mutation is brought about by selective adaptation or (*b*) growing a mixed population of cells in a foreign host permits the process of selective survival of the best adapted cells to take place, cannot be determined with the present data.

There appears, however, to be one fundamental difference between the original observation, reported in 1926 and interpreted in terms of "somatic mutation," and the present data. The original work on transplantation demonstrated that sudden and very significant deviations occurred in the growth capacity of the tumor and that these sudden alterations were *permanent* from the point of origin. In the present data, no sudden or dramatic change took place in the growth capacity of the N-adapted transplants. Certainly the cyclical changes which occurred in the growth capacity of the pBr tumor growing in mice of the N strain is more likely the resultant of some selective process. Again, the stimulated growth capacity of the N-adapted tumor, when placed in pBr mice, was *gradually* acquired over several generations (T_1–T_6) and gradually lost. The stimulated growth capacity of the spindle-cell neoplasm was not permanent but merely of a temporary nature.

However, the two alternative points raised by the present investigation which would be of interest to geneticists or cancer biologists cannot be decided. These are as follows: (*a*) It is not clear whether the change in the transplantability of the spindle-cell neoplasm was of a spontaneous nature or brought about through the process of adaptation. (*b*) The alternative view of "somatic mutation" or a selection of potentially different cancer cells also cannot be decided, but the evidence tends more toward selection.

The change in transplantation, whatever it is or by whatever mechanism or means it is brought about, is probably of a factorial nature.

SUMMARY

Mice (1,948) have been inoculated subcutaneously with a spindle-cell neoplasm which arose originally in the forestomach of a mouse of the pBr descent. The mice used belonged to the pBr and N inbred strains and to the F_1 and F_2 generations of a cross between the pBr and N. Following nine generations of growth in mice of the N strain the tumor (N-adapted) was returned to the original pBr mice for thirteen generations and then into a new group of F_1's and F_2's. The original tumor from the pBr strain gave a 15+ : 1− ratio in a group of F_2 mice. This would indicate that progressive growth is dependent upon either one of two alternative genes. The N-adapted tumor gave a 9+ : 7− ratio expected when two genes are simultaneously needed for progressive *growth* of the tumor. From a dependent tumor the transplant has become more dependent through some change of a genic or factorial nature. The phenomena of adaptation and changes in the transplantation potentials of tumors are discussed.

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A Time Study of the Incorporation of Radiophosphorus into the Nucleic Acids and Other Compounds of a Transplanted Mouse Mammary Carcinoma*

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A previous study (2) with P^{32} and with normal mouse liver tissue indicated the marked metabolic heterogeneity of the pentosenucleic acid (PNA) isolated from various cellular fractions. It was hoped that by extending such a study to tumor tissue we could derive further information concerning possible metabolic interrelationships between the PNA of various fractions and also between PNA and desoxypentosenucleic acid (DNA). Such information should help us to better understand the growth process.

MATERIALS AND METHODS

Supply of tissue.—Such a study required the periodic supply of a uniform tumor tissue over a period of about 1 year. For this purpose a spontaneous mammary carcinoma that arose in an AZF_1 mouse was carried by successive transplantation in AZF_1 or $A_xZ_bF_1$ mice. The transplantation was done by subcutaneous inoculation of a tumor cell suspension to the backs of the recipient animals. After several transplant generations the tumor appeared to have attained a uniform rate of growth which it maintained for 32 generations. The rate of growth was such that tumors of 1–2 gm. could be removed 13–16 days after inoculation.

The radioactive tracer.—At this period, 2 weeks after inoculation, groups of mice[1] containing three or four animals per group were given injections intraperitoneally of a solution containing P^{32} as orthophosphate.[2] The amount of P^{32} used ranged from 0.5 to 2 μc/gm body weight.

Fractionation of tissue.—At time intervals ranging from 15 minutes to 16 hours after injection of P^{32} the mice were sacrificed, and the tumors were homogenized and fractionated as previously described (1, 3, 14). The samples obtained were the nuclear fraction (N), the large granule or mitochondrial fraction (L),[3] the microsomes (M), the ultrasedimentable particles (U), and the nonsedimentable supernatant fluid (S) From each of these fractions the nucleic acids, phospholipids, and "phosphoproteins" were extracted and purified (2, 3, 8, 14). The purified organic compounds and the inorganic phosphorus from an aliquot of the original homogenate (H) were wet ashed and analyzed for P^{32} and total P (2). The nuclear PNA obtained was that previously described (14) as fraction II, which accounts for about 80 per cent of the PNA of mouse mammary tumor nuclei isolated by this citric acid procedure. The preparation of PNA from S has proved more difficult than from the particulates, owing to the larger amounts of protein that fail to coagulate on the first extraction with hot 10 per cent NaCl. Consequently, the alcohol precipitate from this first extract has been re-extracted with hot 10 per cent NaCl and reprecipitated with alcohol. At this point if the PNA is dissolved in water, much of it fails to precipitate with trichloroacetic acid (TCA). Therefore, in about half of the experiments, the second alcohol precipitate was washed with TCA directly and then hydrolyzed with TCA.

The DNA samples have shown an average N:P ratio of 1.63 with a range of 1.60–1.67. The average N:P ratios for the PNA from the cytoplasmic particulates have ranged from 1.75 to 1.79, that for the supernatant PNA 2.35, and that for the nuclear PNA 4–5. Despite the high N:P ratios in some of these fractions, the pentose content based on the orcinol-HCl reaction (measuring purine-bound pentose only), expressed as the percentage of that calculated from the P content, has averaged from 49 to 51.5 per cent for the PNA from the cytoplasmic particulates, 52.5 per cent for the supernatant PNA, and 49.5 per cent for the nuclear PNA. It has been concluded, therefore, that the bulk, if not all, of the P in the PNA samples is nucleic acid P.

RESULTS

In Table 1 are presented the results of the chemical analyses on the whole homogenate of this tumor line and on the separated cellular fractions. It may be seen that the total DNA and the total PNA are very similar in amount and that the bulk of the PNA is found associated with the particulate fractions (primarily M and U) derived from the cytoplasm. The value for S-PNA is probably somewhat low because of the difficulties encountered in quantitatively precipitating this fraction in several experiments. Consequently, two experiments were carried out in which the cytoplasmic extract (after centrifuging the homogenate to remove nuclei) was spun directly at $100{,}000 \times g$ for 1 hour, and all the cytoplasmic particulates

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[1] The animals used in these studies were kindly supplied by Dr. J. J. Bittner.

[2] P^{32} was supplied as H_3PO_4 on allocation from the Oak Ridge National Laboratory.

[3] It was recognized that saline is not ideal for isolation of the mitochondrial fraction, and in most experiments this fraction was discarded. It was retained in a few experiments for comparative purposes only.

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were sedimented together. The sediment and supernate were treated with cold 5 per cent TCA and then with hot TCA to remove the PNA. On the basis of orcinol-HCl tests the PNA P of the combined particulates averaged 493 μg/gm tissue, while that of the supernate averaged 104. This gives a ratio of particulate to supernatant PNA in the cytoplasm of 4.75, a figure that will be made use of later.

Specific activity[4] *of nucleic acid fractions.*—In Table 2 are recorded the specific activities of the inorganic phosphate and of the various nucleic acid fractions. In seven experiments, performed at various time intervals, a large granule or "mitochondrial" fraction was also isolated. In six of these experiments the PNA isolated showed a specific activity that was within the range observed for the PNA from the other particulate fractions of the cytoplasm. It will be noted from Table 2 that the differences in the specific activities of the PNA isolated from the M and U particulate fractions of the cytoplasm are not such as to indicate that these nucleic acids are metabolically distinct. This is particularly evident in the values recorded at the 15- and 30-minute time intervals when the differences might be expected to be most marked. In this tumor tissue, therefore,

TABLE 1

COMPOSITION OF FRACTIONS DERIVED FROM A TRANSPLANTED MOUSE MAMMARY CARCINOMA

Fraction*	Acid-soluble	Inorganic	DNA	PNA	Lipid	Protein residue	Protein residue
			μg P/gm wet weight of tissue				mg N/gm
H	615 (3)† [570–650]	326 (3) [310–340]	834 (28) [720–1,130]	783 (28) [550–910]	301 (2) [284–318]	107 (3) [101–118]	13.9 (5) [12.5–15.5]
N (isolated)			637 (33) [430–760]	59 (35) [33–90]	27 (29) [11–47]	6 (33) [3–14]	3.1 (5) [2.7–3.3]
N (corrected for yield)‡			834	75	35	8	4.7
L				49 (6) [35–75]	97 (7) [59–147]	4 (7) [2–7]	1.3 (5) [0.8–1.9]
M				253 (33) [170–385]	89 (33) [55–123]	6 (33) [2–14]	0.8 (5) [0.5–1.0]
U				193 (33) [130–265]	11 (33) [5–24]	3 (33) [2–5]	0.75 (5) [0.7–0.8]
S	581 (3) [560–620]	301 (3) [290–310]		91 (21) [54–120]	11 (32) [4–24]	9 (32) [5–19]	5.0 (5) [4.0–5.9]
ΣN, L, M, U, S				661	243	30	12.6

* H = homogenate; N = nuclei; L = large granules or "mitochondria;" M = microsomes; U = ultrasedimentable particulates; S = supernatant fluid.

† Values are means of the number of experiments shown in parentheses. Ranges are given in brackets below each mean.

‡ Some nuclei are lost during purification. A correction factor is obtained from the ratio of DNA in the homogenate to that in the isolated nuclei.

TABLE 2

SPECIFIC ACTIVITY (BCC)* OF THE PHOSPHORUS OF VARIOUS NUCLEIC ACID FRACTIONS

Time after P^{32} injection (hours)	Inorganic *i*†	DNA *d*	N-PNA *a*	M-PNA	U-PNA *c*	S-PNA *b*
0.25	7,470 (5)‡ [4,900–9,300]	5.5 (5) [2.1–10.1]	131 (5) [65–216]	3.3 (5) [1.1–6.0]	3.9 (5) [1.0–8.8]	24.6 (3) [16–38]
0.5	10,400 (4) [8,600–11,600]	27.4 (4) [22–36]	633 (4) [540–760]	14.1 (4) [11–18]	15.2 (4) [11–22]	62 (3) [50–84]
1	9,640 (4) [8,000–11,700]	84.5 (4) [64–110]	1,680 (4) [1,470–2,170]	69.6 (3) [48–104]	80.8 (4) [49–117]	179 (4) [130–220]
2	6,940 (4) [6,100–8,000]	220 (4) [200–253]	2,690 (4) [2,370–3,370]	260 (4) [231–290]	242 (4) [212–265]	344 (4) [284–415]
3	4,920 (4) [3,300–6,400]	365 (4) [260–414]	2,840 (4) [2,100–3,700]	461 (4) [409–519]	450 (4) [345–522]	595 (4) [354–767]
4	4,130 (4) [3,200–4,700]	530 (4) [512–575]	3,000 (4) [2,480–3,380]	649 (4) [563–685]	582 (4) [566–594]	891 (4) [770–1,000]
8	2,670 (4) [2,500–3,000]	901 (4) [778–1,040]	2,360 (4) [2,220–2,650]	1,110 (4) [1,020–1,210]	1,010 (4) [853–1,110]	1,150 (4) [974–1,440]
16	1,610 (3) [1,530–1,700]	1,160 (3) [1,100–1,230]	1,640 (3) [1,630–1,650]	1,520 (3) [1,470–1,590]	1,360 (3) [1,350–1,370]	1,210 (3) [1,180–1,260]

* See footnote 4.

† *i*, *d*, *a*, and *b* are symbols subsequently used for inorganic P, DNA P, nuclear PNA P, and supernatant PNA P, respectively. *c* is the symbol used for the particulate PNA P, i.e., the P of M-PNA plus U-PNA.

‡ Values are means of the number of experiments shown in parentheses. Ranges are given in brackets below each mean.

[4] Throughout this paper specific activity is expressed as the Biological Concentration Coefficient (BCC) as suggested by Schulman and Falkenheim (12). It is defined as follows:

$$\text{BCC} = \frac{(\text{counts/min/mM P}) \times 100}{\text{counts/min administered/gm body weight}}.$$

The relative specific activities reported in a previous paper (2) may be converted to BCC by multiplying them by 101.

it appears reasonable to visualize all the PNA of the cytoplasmic particulates (P-PNA) as being metabolically homogeneous. In sharp contrast to such homogeneity in tumor are some recent unpublished results obtained with normal liver tissue in which the PNA isolated from the U fraction 15 minutes after administration of P^{32} had 4–5 times the specific activity of the M-PNA.

TABLE 3

SPECIFIC ACTIVITY (BCC)* OF THE PHOSPHORUS OF LIPID AND PROTEIN RESIDUE FRACTIONS

TIME AFTER P^{32} INJECTION (HOURS)	LIPID		PROTEIN RESIDUE
	M and U	S	M, U, and S
0.25	17 (9)†	99 (3)	830 (11)
	[10–26]	[41–118]	[270–1,570]
0.5	54 (6)	187 (3)	1,480 (11)
	[42–66]	[83–302]	[930–2,110]
1	150 (8)	284 (3)	2,380 (11)
	[107–186]	[190–430]	[1,280–3,040]
2	377 (8)	486 (4)	2,480 (12)
	[313–433]	[458–525]	[1,690–3,360]
3	607 (8)	771 (2)	2,400 (11)
	[485–673]	[557–985]	[1,450–3,630]
4	864 (7)	995 (4)	2,380 (12)
	[760–910]	[840–1,080]	[1,140–2,970]
8	1,220 (8)	1,350 (4)	1,990 (11)
	[1,030–1,430]	[1,130–1,600]	[1,400–2,670]
16	1,470 (6)	1,520 (3)	1,700 (8)
	[1,400–1,530]	[1,410–1,700]	[1,510–1,950]

* See footnote 4.

† Values are means of number of observations shown in parentheses. Ranges are shown in brackets below each mean.

It will be observed that the nuclear PNA shows by far the most rapid increase in specific activity of all the nucleic acid fractions isolated. This is similar to previous observations for normal liver tissue (2). Initially, the S-PNA has a specific activity much greater than that of the particulate PNA, but the latter rapidly approaches it.

Specific activity of phospholipid and "protein residue" fractions.—Table 3 shows the specific activities of the phospholipid P and the "protein residue" P. Since the phospholipid P of the M and U fractions showed no significant differences, these are reported together. Similarly, the phospholipid P of the "mitochondrial" fraction obtained in seven experiments showed no differences from that of the M and U fractions. On the other hand, the S-phospholipid P shows a significantly higher specific activity at early time intervals, but these values are approached by the particulate phospholipid values within 16 hours. The nuclear phospholipid has shown a specific activity of approximately 85 per cent of that of the cytoplasmic particulates.

The "protein residue" P shows an extremely rapid rise in specific activity, and no significant differences have been observed between any of the cytoplasmic fractions studied. Again, however, the nuclear "protein residue" P has shown a specific activity of approximately 85 per cent of that of the other fractions.

Chart 1 illustrates the time course of the specific activities of several of these fractions and demonstrates that by 16 hours they have very nearly reached equilibrium.

DISCUSSION

The specific activity of the "protein residue" P shows an extremely rapid increase, comparable to the few observations previously made on this fraction from liver (2). An inspection of Chart 1 indicates that the curve, though rising more rapidly than that for the N-PNA, goes through its maximum at a value well below that for N-PNA. This seems to imply a heterogeneity of the P in this fraction (10)[5] with one part incorporating P^{32} very rapidly and a second part more slowly. Even the slower fraction, however, has come to equilibrium by 16 hours.

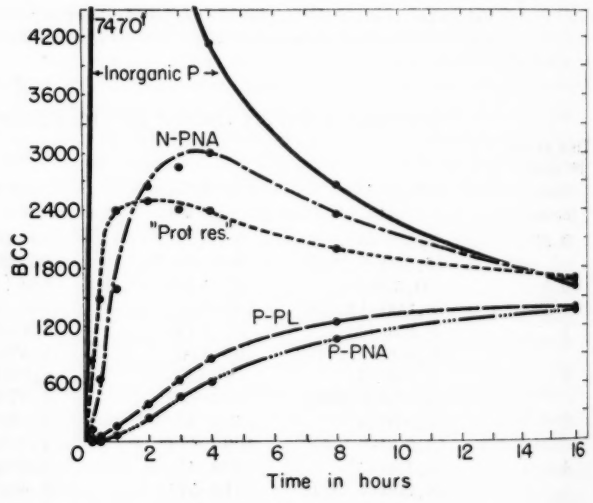

CHART 1.—Specific activity-time curves for inorganic P, nuclear PNA (N-PNA), "protein residue," particulate phospholipid (P-PL), and particulate PNA (P-PNA).

BCC refers to the specific activity. See footnote 4.

The particulate phospholipid incorporates P^{32} much less rapidly than the same fraction in liver (2). However, in tumor tissue the supernatant phospholipid shows a higher specific activity than the particulate phospholipid, whereas in liver no differences were observed.

Analysis of the specific activity-time curves for nucleic acid fractions.—Our particular interest has been focused on the incorporation of P^{32} into the

[5] Kennedy (10), in an oral report, presented evidence for the isolation, from a protein residue fraction, of serine and threonine phosphates with specific activities several times that for the total P of this fraction.

various nucleic acids of the cells. One of our objectives was to find a tissue in which growth was sufficiently reproducible and constant over the experimental period that we could derive some useful information by following the time-course of the specific activity of the DNA and of the various PNA fractions.

One important simplifying assumption which is basic to all the calculations that follow was that even this rapidly proliferating tumor tissue could, as a first approximation, be visualized as a tissue in a steady state. This assumption was made since it seemed probable, on preliminary inspection of the curves, that the rates at which most of the constituents were equilibrating with their precursors greatly overshadowed the rate of growth. In view of the results reported in this paper the assumption of a steady state appears to have been a valid approximation.

Interrelationships between PNA fractions.—On the basis of previous observations (2) on mouse liver tissue a tentative hypothesis was suggested which proposed that some unknown intermediate, x, which equilibrates very rapidly with inorganic P, i, is the immediate precursor of the phosphorus of nuclear PNA, a, and also of the cytoplasmic supernatant PNA, b, which in turn is the precursor of the particulate PNA, c, of the cytoplasm. Such a scheme can be pictured as follows:

$$\begin{array}{l} \rightarrow \text{Inorg. P} \rightleftharpoons x \rightleftharpoons \text{N-PNA} \\ \qquad\qquad\quad \downarrow \qquad\quad \uparrow\downarrow \\ \qquad\qquad\quad \text{S-PNA} \rightleftharpoons \text{P-PNA} \end{array} \quad \text{or} \quad \begin{array}{l} \rightarrow i \underset{2}{\overset{1}{\rightleftharpoons}} x \underset{4}{\overset{3}{\rightleftharpoons}} a \\ \qquad\qquad\quad \downarrow \qquad\quad \uparrow\downarrow \\ \qquad\qquad\quad b \underset{6}{\overset{5}{\rightleftharpoons}} c \end{array} \quad . \quad (1)$$

The justification for considering the particulate PNA as the end of the reaction chain—thus permitting the formulation of equation (4) below—is that at early time intervals no P fraction that we have isolated from this tissue has shown a lower specific activity than the P-PNA. Since the major P fractions have been analyzed, it seems improbable that P-PNA could be the immediate precursor of any other appreciable P pool, which by a reversible reaction might affect its specific activity-time curve.

Since the steady state is assumed in scheme 1, then v_1 (the rate at which P in compound x is incorporated into compound a) is equal to v_2 (the rate at which P returns from a to x). Similarly, $v_3 = v_4$ and $v_5 = v_6$. As previously described (2), one can define the time changes in the specific activities of compounds a, b, and c by means of the following equations:

$$\frac{dA}{dt} = \frac{v_1}{a}(X - A) = k(X - A) \quad (2)$$

$$\frac{dB}{dt} = \frac{v_3}{b}(X - B) - \frac{v_5}{b}(B - C) = l(X - B) - n(B - C) \quad (3)$$

$$\frac{dC}{dt} = \frac{v_5}{c}(B - C) = m(B - C) \; . \quad (4)$$

In these equations a, b, and c refer to the μg PNA P/gm wet tissue to be found in nuclei, cytoplasmic supernate, and cytoplasmic particulates, respectively, while X, A, B, and C refer to the specific activities of the compounds x, a, b, and c, respectively. The constants k, l, m, and n define that fraction of a given compound replaced each hour. Thus, k is that fraction of the P present in a which traverses the path from x to a each hour.

Metabolic reactions involving DNA.—In attempting to analyze the data with respect to DNA we have made the assumption that the incorporation of P^{32} into DNA occurs only in cells that are preparing to undergo division, i.e., that in most of the cells at any given time the DNA is metabolically inert as far as its P is concerned. This assumption rests on the observation of Osgood *et al.* (11) that in three nondividing tissues (skeletal muscle, cartilage, and brain cortex) of an adult patient treated with P^{32} for 1 year the DNA showed no incorporation of P^{32}; on our observation (2) that the DNA of adult mouse liver cells incorporates extremely little P^{32}; and on the observation of Brues *et al.* (5) that when P^{32} is incorporated into liver during regeneration it remains in the DNA for a long time, whereas it gradually leaves the other P compounds of the liver.

In a cell that is preparing to undergo division and is synthesizing an equivalent amount of new DNA, one could visualize several possibilities: (*a*) the pre-existing DNA of the mother cell might remain metabolically inert, and therefore only half of the DNA of the daughter cells would become labeled; (*b*) the pre-existing DNA might go completely into the pool of its precursors from which a double amount of DNA, all labeled, would emerge; or (*c*) the pre-existing DNA might enter into reversible reactions with its labeled environment at, or at about, the same time that an equivalent amount of new DNA was being synthesized from the precursor pool. Regardless of which of these three possibilities is correct, the rate of incorporation of P^{32} into DNA will be a function of the rate of synthesis of DNA. Furthermore, since we have found no significant differences in the content of DNA/gm of tissue between small and large tumors, one can also conclude that the incorporation of P^{32} is a function of tumor growth.

We may visualize a general reaction scheme as follows:

$$\rightarrow \text{Inorg. P} \rightleftarrows x \rightleftarrows \text{DNA} \quad \text{or} \quad \rightarrow i \rightleftarrows x \underset{8}{\overset{7}{\rightleftarrows}} d \qquad (5)$$

where v_7 is the rate at which P goes from a precursor, x, to DNA, d, and v_8 is the rate of a possible reverse reaction. This system is not in a steady state, and we know that $v_7 > v_8$. If possibility (*a*) above is correct, then $v_8 = 0$; if possibility (*b*) is correct, then $v_8 = v_7/2$; and if possibility (*c*) is correct, then $0 < v_8 < v_7/2$.

The quantity $(v_7 - v_8)/\boldsymbol{d}$ represents the fractional increase of DNA per unit time and will be referred to as the growth constant, g. If one assumes that the number of cells in which DNA is being synthesized is proportional to the number of cells present (or to the total DNA present) at any given time, and that this proportionality remains constant over the experimental period of 16 hours,[6] then one can write that $\boldsymbol{d} = \boldsymbol{d}_0 e^{gt}$ where $\boldsymbol{d}_0$ is the amount of DNA at time zero, $\boldsymbol{d}$ the amount of DNA at any specified future time, g the growth constant, and t the time in hours. Using this assumption of the constant percentage increase in DNA over the experimental period, one can derive the following equation defining the time change in the specific activity of DNA:

$$\frac{dD}{dt} = \frac{v_7}{\boldsymbol{d}}(X - D) = f(X - D) = (g + h)(X - D) \qquad (6)$$

where

$$f = \frac{v_7}{\boldsymbol{d}}; \quad g = \frac{v_7 - v_8}{\boldsymbol{d}}; \quad h = \frac{v_8}{\boldsymbol{d}}; \quad \text{and} \quad 0 \leq h \leq g\,.$$

It is surprising at first to find the form of the equation identical to that for reversible, steady-state systems, but this is a direct result of the assumption that the rate at which DNA is formed in the tissue is proportional to the amount there.

Proposed interrelationships between DNA and the various PNA fractions.—With equation (6) one can readily demonstrate that neither inorganic P nor any of the fractions of PNA P isolated can fulfill the role of x. Thus, as an initial assumption to be tested, we have visualized the following:

$$\begin{array}{ccccc} & & \text{DNA} & & \\ & & \uparrow\downarrow & & \\ \rightarrow \text{Inorg. P} & \rightleftarrows & \boxed{x} & \rightleftarrows & \text{N-PNA} \\ \downarrow & & \uparrow\downarrow & & \\ & & \text{S-PNA} & \rightleftarrows & \text{P-PNA} \end{array} \qquad (7)$$

or

$$\begin{array}{ccccc} & & d & & \\ & & {\scriptstyle 7}\uparrow\downarrow{\scriptstyle 8} & & \\ \rightarrow i & \rightleftarrows & \boxed{x} & \underset{2}{\overset{1}{\rightleftarrows}} & a \\ \downarrow & & {\scriptstyle 4}\uparrow\downarrow{\scriptstyle 3} & & \\ & & b & \underset{6}{\overset{5}{\rightleftarrows}} & c \end{array}\,.$$

In words, this assumption says that the phosphorus of the DNA, d, of the nuclear PNA, a, and of the cytoplasmic supernatant PNA, b, is all derived from precursors that share a common metabolic box. It is possible that the phosphorus precursors of each of these nucleic acids are identical, though this is not necessary and does not seem very probable. If the precursors of a, b, and d are so rapidly equilibrated among themselves that their specific activities are indistinguishable, then for our subsequent considerations x may be considered a single substance.

Evaluation of proposed mechanism.—The first step in testing the above hypothesis involves analysis of the postulated chain of reactions from x to b to c as shown in equation (7). Calculations on the cytoplasmic constituents have been carried through the 8-hour interval. From Chart 2 it may be seen that it was possible to draw a smooth curve through, or very close to, all the experimental points for C, i.e., the points for the specific activity of the particulate PNA of the cytoplasm. In the case of the supernatant PNA, the experimental points for specific activity, B, were less consistent, and a smooth curve, B_1, was drawn among the points as a first approximation. At this stage the areas under curves B_1 and C (i.e., β_1 and γ, respectively) were evaluated and introduced into the integrated form of equation (4):

$$C = m(\beta_1 - \gamma)\,. \qquad (8)$$

By plotting C against $(\beta_1 - \gamma)$, it was possible to draw the best straight line through the points. The slope of this line would represent the most probable value of m, which was evaluated as 1.18. Using this value for m, along with the fixed values for C and γ, equation (8) can be used to re-evaluate β, this time calling it β_2. Finally, the values for β_2 at the various time intervals may be numerically differentiated, and a smooth curve drawn through these differentials to give B_2 as shown in Chart 2. It may be seen, then, that a curve B_2, varying only a little from the curve B_1 first drawn among the experimental points, will fit exactly into the role of precursor for the particulate PNA.

[6] The validity of the assumption of a constant mitotic rate throughout the 16-hour experimental period is strengthened by two sets of observations. Several observers (4, 6) have noted that for malignant tumors (epidermoid carcinoma and carcinoma of large intestine have been investigated) the mitotic rate remains constant and does not show the diurnal variation seen in normal tissues and in regenerating tissues. Unpublished observations in this laboratory indicate the lack of any significant difference in the rate of incorporation of P^{32} into the DNA of a mouse mammary carcinoma when tested between 7 and 9 A.M. as against 9–11 P.M.

The next step involves writing equations (6), (2), and (3) in their integrated forms.

$$D = f(\xi - \delta) \tag{9}$$

$$A = k(\xi - \alpha) \tag{10}$$

$$B = l(\xi - \beta) - n(\beta - \gamma). \tag{11}$$

In these equations α, β, δ, and ξ represent the integrals of A, B, D, and X, i.e., they represent the areas under the respective specific activity-time curves up to any given point. Since $(\beta - \gamma) = C/m$, as seen from equation (8), and since $n/m = c/b = 4.75$[7] as seen from equations (3) and (4), equation (11) may be written as

$$B = l(\xi - \beta) - 4.75C. \tag{11a}$$

Finally, equation 11*a* may be rearranged to read

$$B + 4.75C = \bar{B} = l(\xi - \beta). \tag{11b}$$

The hypothesis to be tested assumes that ξ is identical in equations (9), (10), and (11*b*). Thus, from equations (10) and (11*b*) we have

$$\xi = \alpha + k^{-1}A = \beta + l^{-1}\bar{B}. \tag{12}$$

Dividing through by A and rearranging we get

$$\frac{(\alpha - \beta)}{A} - l^{-1}\frac{\bar{B}}{A} = -k^{-1}. \tag{12a}$$

Similarly, from equations (9) and (10) we get

$$\frac{(\alpha - \delta)}{D} + k^{-1}\frac{A}{D} = f^{-1}. \tag{13}$$

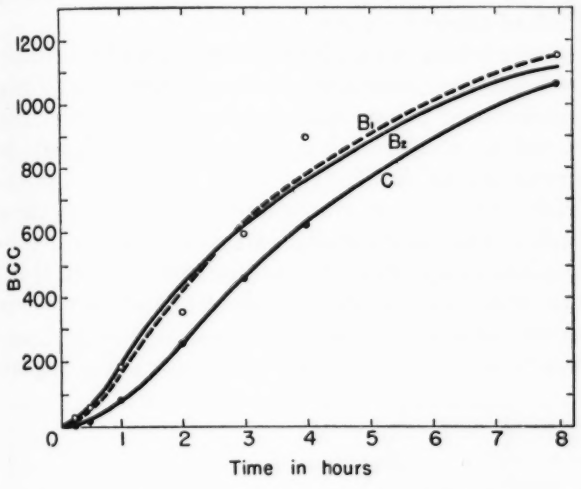

CHART 2.—Specific activity-time curves for the supernatant PNA (B) and the particulate PNA (C) of the cytoplasm.

B_1 was first smoothed curve drawn among the B points while B_2 defines precisely the time course of a fraction that could be the immediate precursor of the particulate PNA.

BCC refers to the specific activity. See footnote 4.

If we now plot $\bar{B}/A$ against $(\alpha - \beta)/A$ and A/D against $(\alpha - \delta)/D$, as shown in Charts 3 and 4, we can evaluate l^{-1}, k^{-1} and f^{-1} from the proper slopes and intercepts, provided the points can reasonably be considered to fall on straight lines. Since k^{-1} must be the same for both plots, then the selection of best values for all three constants is influenced

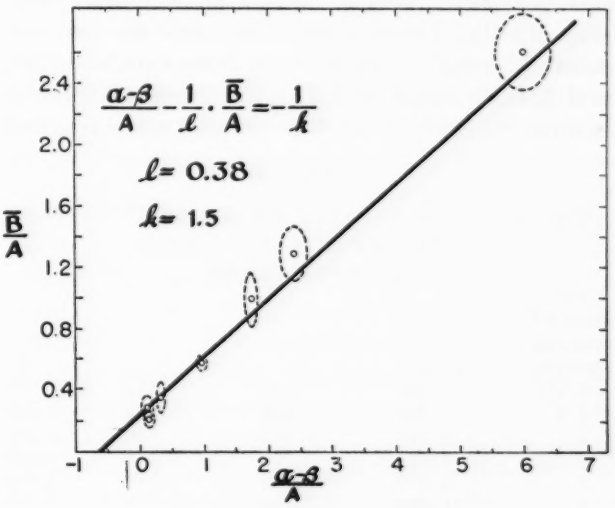

CHART 3.—Graphical solution of equation (12*a*).

Errors of $\bar{B}/A$ calculated as $\sqrt{[\Sigma(x - \bar{x})^2]/(N - 1)}$ where x is the ratio of $\bar{B}/A$ in a given experiment, $\bar{x}$ the mean $\bar{B}/A$ ratio at a given time point, and N the number of observations at a given time point ($N = 3$ or 4).

Errors of $(\alpha - \beta)/A$ are crudely estimated on the basis of the assumption that a value of A higher than the value at that point on the smoothed curve is associated with the value of α proportionately higher than the α derived from the smoothed curve. Similar reasoning applies to B and β. This allows the calculation of α and β for each observed value of A and B, and therefore gives three or four "observations" of the ratio $(\alpha - \beta)/A$ for each time point and allows the estimation of a standard deviation as above.

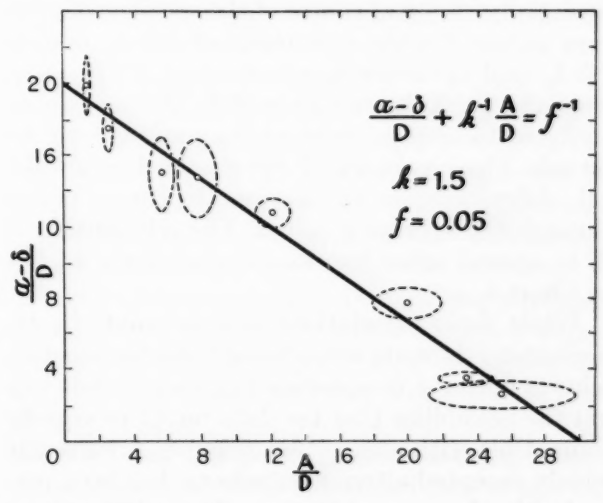

CHART 4.—Graphical solution of equation (13). See Chart 3 for method of estimating errors.

[7] 4.75 is the ratio of the particulate PNA in the cytoplasm to the supernatant PNA. See first paragraph under "Results."

by all the experimental data, thus minimizing errors inherent in the data for any single time curve. The values for l, k, and f, as evaluated by this graphical method, are 0.38, 1.5, and 0.05, respectively. Using these constants we can calculate ξ_D from equation (9), ξ_A from equation (10), and ξ_B from equation (11a) and compare them as is done in Table 4. In this calculation the actual

TABLE 4

CALCULATED AREAS UNDER SPECIFIC ACTIVITY-TIME CURVES OF POSTULATED NUCLEIC ACID PRECURSORS

TIME AFTER P^{32} INJECTION (HOURS)	ξ_D*	ξ_A	ξ_B	X†
0.25	110	102	112	720
0.5	551	519	364	1,850
1	1,720	1,800	1,510	2,700
2	4,570	4,770	4,430	3,220
3	7,770	7,760	8,170	3,440
4	11,500	10,900	11,700	3,250
8	21,900	21,200	21,800	2,090
16	35,400	36,400		1,640

* ξ_D, ξ_A, and ξ_B calculated from equations 9, 10, and 11a respectively. Values of the constants, obtained from the graphical solution, were 1.5, 0.05, and 0.38 for k, f, and l, respectively. Values of A, B, C, and D used were those experimentally observed, but values of the areas α, β, and δ were obtained from the smoothed time curves of A, B, and D.

† X is derived by numerical differentiation of the time curve of the average ξ values.

experimental values for A, B, C, and D at the various time points were used, but it was necessary to use the areas α, β, and δ derived from the smoothed curves. Inspection of Table 4 shows that the ξ values calculated from the three reaction pathways are in good agreement, indicating the essentially identical course of the specific activity-time curves for the precursors of DNA, nuclear PNA, and cytoplasmic supernatant PNA. Also, shown in Table 4, are values of X, the specific activity of these precursors at the various time intervals. These values of X are obtained by graphical differentiation of a smooth curve drawn through the average ξ values. The relationship of X to several other fractions is shown graphically in Chart 5.

While these calculations indicate that the experimental data are compatible with the reaction scheme outlined in equation (7), they do not rule out the possibility that the data might be equally compatible with some other scheme. A currently widely accepted alternative scheme has been presented by Jeener and Szafarz (9), and they have marshalled the arguments supporting it. In this scheme it is proposed that nuclear PNA is a precursor of the nonsedimentable PNA of the cytoplasm. If the values of $B + 4.75C$ are plotted against $(\xi - \beta)$, it may be seen from equation (11b) that a straight line of slope l should result. Such a plot, using the average ξ values derived above, gives an excellent straight line passing through the origin. Regardless of what the precursor of this cytoplasmic PNA (B) might be, if the area under its curve were substituted for ξ in equation (11b) and a similar plot made, a straight line through the origin should result. However, when the values for α, the area under the nuclear PNA curve, were substituted for ξ, the resulting plot described a curve for which the points were very far from falling on a straight line. Thus, at least in this tissue, the nuclear PNA isolated cannot be considered the precursor of the supernatant PNA of the cytoplasm.

Comparison of f *with the growth rate of the tumor.* —Of all the constants that emerge from these calculations, f has special interest because it is related to the growth constant, g, which can be approximated by independent means. Unfortunately, the tumor transplant line used in these studies was accidentally lost before several contemplated experiments could be undertaken. However, three independent evaluations of increase in tumor volume by caliper measurements had previously been done on this tumor for other experiments. Two of these evaluations involved twenty and thirteen tumors, respectively, that had been transplanted to the thigh regions (14), while the third one involved 24 tumors transplanted to the back. In these evaluations the tumor volume was crudely estimated as the volume of a sphere whose diameter was the average of the two external dimensions of the tumor minus double the thickness of the skin. In the first experiment, the crude growth rate per hour was 2.0 per cent between the

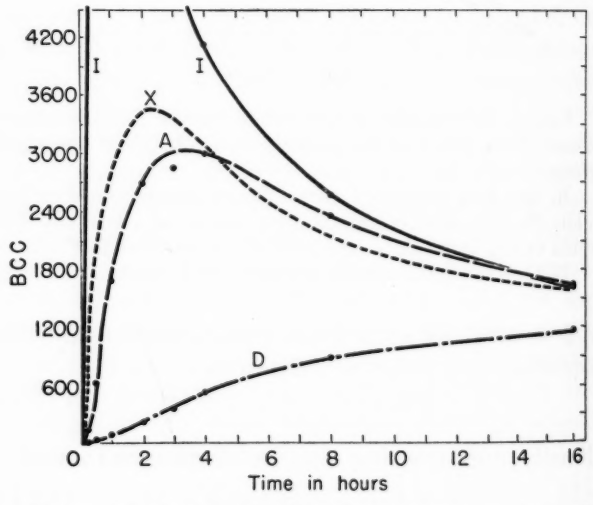

CHART 5.—Specific activity-time curves for inorganic P (I), nuclear PNA (A), DNA (D), and a postulated precursor (X).

BCC refers to specific activity. See footnote 4.

12th and 17th days, in the second experiment 2.0 per cent between the 13th and 18th days, and in the last experiment 2.6 per cent between the 9th and 12th days.

A growth rate of 5 per cent per hour, which was the calculated value of f, implies that the tumor should triple its size each 24 hours, and this tumor was clearly not growing that fast. From the crude estimation of its actual growth rate, g, it would appear that this might be one-half of f. Referring back to equation (6) it would appear clear then that v_8/d or h does have a finite value and that the best approximation currently possible is that it is equal to g. Thus, it is tentatively concluded that, in a cell preparing to synthesize new DNA, the pre-existing DNA does enter into metabolic reactions with its environment and that this pre-existing DNA goes completely (so far as its P is concerned) into the metabolic pool of its precursor.

This same conclusion has been recently reached by Stevens, Daoust, and Leblond (13) from studies on rat liver and intestine in which they compared the "rate" of incorporation of P^{32} into DNA to evaluations of the rate of new cell formation in these tissues. They calculate the rate of incorporation of P^{32} on the assumption that the precursor of DNA has a specific activity-time course which can be represented by that for the acid-soluble P of their tissues. Their values on the rate of incorporation are expressed as DNA specific activity at the time of sacrifice as a percentage of the "average specific activity" of the acid-soluble P from the time of injection to that of sacrifice. They do not explain how they calculate the average specific activity of the acid-soluble P, but from their data one can calculate[8] that the value they use for the average activity from 0 to 3 hours is the observed value at 3 hours and that the value they use for the average from 0 to 6 hours is the mean of the observed values at 3 and 6 hours. From our previous study (2) and from unpublished data pertaining to acid-soluble P obtained in the same study, we can calculate that for mice injected intraperitoneally with P^{32} the observed specific activity of liver acid-soluble P at 3 hours is 73 per cent of the average activity over the 3 hours. We assume that a somewhat comparable relation exists for the liver acid-soluble P in their rats which were injected subcutaneously. Furthermore, their calculation of the 6-hour average tacitly assumes that the average between 3 and 6 hours is equal to the value observed at 6 hours, rather than to some value intermediate between the 3- and 6-hour values as would be necessary on this descending portion of their curve. We have recalculated their data, assuming that the 3-hour value for acid-soluble P should be divided by 0.73 to give the average over the first 3 hours, and using, as a first approximation, the mean of any two adjacent values as the average activity throughout that time interval. On this basis we find that their liver DNA at 24 hours has a specific activity that is 1.11 per cent that of the average for acid-soluble P instead of 1.36 per cent as they report. This makes what they call the "rate of formation of DNA" considerably less than twice the rate of new cell formation which they give as 0.71 per cent per day. On the other hand, the observations reported in this paper indicate that the postulated precursor of DNA in mammary tumor tissue has a specific activity-time course well below that for inorganic P (and also below that for total acid-soluble P) throughout the first 8 or 12 hours. This implies that if Stevens *et al.* had been able to calculate the average specific activity of the actual precursor they would have obtained a value for DNA formation greater than 1.11 per cent per day and probably in the range of double the rate they report for new cell formation—as they claim their study demonstrates. It would appear that they reached their conclusion because of a canceling of errors.

A similar recalculation of their data on intestinal DNA leads to a value for DNA at 3 hours of 10.75 per cent that of the recalculated average activity of acid-soluble P. This may be expressed as 86 per cent per day, whereas they report 117.6 per cent. Again, one is justified in assuming that the actual immediate precursor of the P in intestinal DNA has an average specific activity over the first 3 hours that is substantially below the average for acid-soluble P and would thus lead to a calculation of DNA formed per day substantially greater than 86 per cent. In mouse mammary tumor the average specific activity of the postulated DNA precursor during the first 3 hours is only 50 per cent that of the acid-soluble P during the same time. If this same correction factor should apply to intestine, it would imply a daily rate of DNA formation of 170 per cent, thus making it much more than twice their calculated rate of new cells formed per day of 52.7 per cent. Whether this discrepancy—(*a*) results from the possibility that the correction factor to be applied to intestine should be much less than would apply in mammary carcinoma, (*b*) indicates need for re-evaluation of the 52.7 per cent per day of cell increase which was based on the piling up of cells in mitosis over a

[8] A personal communication from Dr. Leblond confirms that the calculation described here is equivalent to the one used by them.

4-hour period after the administration of colchicine, or (*c*) implies that the thesis advanced by us as well as by Stevens, Daoust, and Leblond is invalid—must await the accumulation of additional experimental evidence.

This conclusion, that the pre-existing DNA of a cell that is preparing to divide does incorporate P^{32}, is also consistent with the suggestion made by v. Euler and Hevesy (7) and with the data presented in a previous paper by the present authors (14). In our studies on mouse mammary carcinoma it was observed that a dose of x-radiation, sufficient to cause complete cessation of tumor growth and complete inhibition of new cells entering mitosis, inhibited the incorporation of P^{32} into the DNA to only 50 per cent of that seen in the non-irradiated control tissue. It was proposed that x-radiation blocked the net synthesis of DNA but did not affect the "turnover" of the pre-existing DNA.

TABLE 5

CONSTANTS FOR REACTIONS SHOWN IN EQUATIONS (7), (8), (9), (10), AND (11)

μg NA P/gm wet tissue	Reaction involved	Fractional turnover of product/hr	Rate of reaction (μg P/gm/hr)
$a = 75$	$x \rightarrow a$	$k = 1.5$	$v_1 = 113$
$b = 104$	$x \rightarrow b$	$l = 0.38$	$v_3 = 40$
$c = 495$	$b \rightarrow c$	$m = 1.18$	$v_5 = 580$
	$c \rightarrow b$	$n = 5.6$	$v_6 = 580$
$d = 834$	$x \rightarrow d$	$f = 0.05$	$v_7 = 42$
	$d \rightarrow x$		$v_8 = 21$

Significance of constants and implications concerning steady state.—The values for the various constants together with the amount of P in each of the nucleic acid fractions, as taken from Table 1, enable us to calculate the rate at which P is passing via the various pathways in equation (7). These rates are recorded in Table 5.

As may be seen in Table 5, the amount of nucleic acid P, in this case presumably intact PNA, that is going back and forth each hour between a nonsedimentable form in the supernate (*b*) and the particulate lipoprotein-nucleoprotein form (*c*) is 75 per cent of all the PNA P in the cell. It shows also that the fractional turnover between S-PNA and P-PNA is nearly as great as that between precursor and N-PNA, and should make one cautious about concluding that a certain fraction is metabolically less active than some other merely because its specific activity is much lower. Table 5 also appears to justify the original approximation that this system could be treated as a steady state, since even the slowest reaction ($x \rightleftharpoons b$) has a turnover of 38 per cent/hour where the net increase due to growth is of the order of 2–2.6 per cent/hour.

SUMMARY AND CONCLUSIONS

Mice bearing a transplanted mammary carcinoma have been sacrificed at time intervals varying from 15 minutes to 16 hours after the intraperitoneal injection of P^{32}. The tumors have been fractionated by differential centrifugation to yield nuclei (N), microsomes (M), an ultrasedimentable particulate fraction (U), and a supernatant fluid (S). From each of these fractions the nucleic acids, phospholipids, and "protein residues" have been isolated and the specific activities of the P of these constituents measured along with the specific activity of the inorganic P derived from the whole homogenate.

Next to inorganic P, of those fractions studied the "protein residue" P showed the most rapid initial rise in specific activity. However, the shape of its specific activity-time curve leads us to conclude that it is a heterogeneous fraction with respect to its P, part of which equilibrates very rapidly with inorganic P and the rest more slowly. Nevertheless, it has all equilibrated by 16 hours. No significant differences were observed in the specific activities of this "protein residue" P derived from any of the cytoplasmic fractions, but the "protein residue" P from the nuclei consistently showed a slightly lower specific activity.

The phospholipid P from the cytoplasmic particulate fractions was indistinguishable on the basis of specific activity. However, the supernatant phospholipid P showed a significantly higher specific activity at early time intervals but its time curve was approached by the curve of the particulate phospholipid P as both of them approached equilibrium with the inorganic P at 16 hours. Again, the nuclear phospholipid P consistently showed a slightly lower specific activity than that from the other fractions.

The pentosenucleic acids could be placed in three metabolically distinct fractions—the nuclear (N-PNA), the cytoplasmic supernatant (S-PNA), and the cytoplasmic particulate (P-PNA). The specific activity-time curves for these three fractions and for the DNA were consistent with the assumption that the P of DNA, N-PNA, and S-PNA came from precursors whose specific activities had essentially identical time courses. This is interpreted to mean that the immediate precursors (perhaps a mixture of desoxypentose- and pentose-mononucleotides) were all in rapid equilibrium with some common P donor (such as ATP). The data were also consistent with the assumption that S-PNA was the immediate precursor of P-PNA. On the

other hand, the data were not consistent with the prevalent assumption that N-PNA is the precursor of either fraction of cytoplasmic PNA.

Turnover constants, expressed in terms of that percentage of the P in a product which passes from precursor to product each hour, were calculated as 150 per cent for N-PNA, 38 per cent for S-PNA, and 118 per cent for P-PNA. In the case of DNA, the amount of P incorporated per hour was calculated as 5 per cent of that present, whereas the net increase of DNA was estimated to be 2–2.6 per cent/hour. This led to the tentative conclusion that, in a cell preparing to synthesize new DNA, the pre-existing DNA enters into metabolic reactions with its environment and that this preformed DNA goes completely (so far as its P is concerned) into the metabolic pool of its precursor.

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Disappearance of Natural Heteroagglutinins for Human Erythrocytes from the Sera of Rats with Progressively Growing Tumors*

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Current studies on the serological activity of normal and tumor-bearing rats show a striking difference in the reaction of their sera to suspensions of human red blood cells; the sera of normal rats caused agglutination of human erythrocytes, while the sera from tumor-bearing rats failed to do so.

Findings of earlier investigators (4, 5, 9, 11–13) indicated that neoplastic growths caused an alteration of the immunological response of man and of lower mammals. To the best of our knowledge, no one has investigated heteroagglutinins for human blood cells at different stages of homologous neoplastic growth. It seemed important, therefore, to study the agglutinins for heterologous blood cells present in sera of normal and tumor-bearing animals. The results of these studies are reported below.

MATERIALS AND METHODS

Normal rats, of both sexes, were selected from two highly inbred strains, the P.A.[1] and the Lewis[2] strains. In some of them a small portion of viable tumor tissue was later implanted. The implanted tumors grew progressively, causing death of the hosts after 25–35 days.

The transplanted tumors used were sarcomas 231, 6, 3 and 10; carcinoma 5 and lymphoma 8. Sarcomas 231 and 6 were originally induced in rats of the P.A. strain by the injection of methylcholanthrene. Carcinoma 5 originated spontaneously in a rat of the P.A. strain. Sarcomas 10 and 3 were induced in rats of the Lewis strain by methylcholanthrene, and lymphoma 8 arose spontaneously in an old breeding female of the Lewis strain. All these neoplasms proved to be 100 per cent transplantable in rats of their strain of origin. Other rats tested had primary tumors that developed in the host following injections of methylcholanthrene in mineral oil. These rats were used when the tumors had grown to a moderately large size—about 6 months after injection of methylcholanthrene.

Two and one-half per cent suspensions of human group O and group AB blood cells were used to test the antihuman agglutinin titers in rat sera. Blood from the same two human donors (Rh positive), drawn by venipuncture, was used throughout for the preparation of red cell suspensions. Blood to be used for suspensions was drawn without an anticoagulant and mixed immediately with 20 volumes of 0.9 per cent sodium chloride solution. Since washing and resuspensions of the cells did not appear to affect their sensitivity to agglutination, this procedure was omitted. Erythrocyte suspensions were discarded after 14 days (maximum limit), since hemolysis, which occurred upon standing, seemed to reduce their sensitivity to agglutination.

One ml. of blood was collected from each rat by cardiac puncture with a 1-ml. syringe and a 22-gauge needle, care being taken to avoid aspiration of tissue or pleural fluid. After separation from the clot, the clear, slightly amber serum was inactivated at 55°–56° C. for 30 minutes.

Hemagglutination test.—The hemagglutination test was based upon the rather simple method of blood typing. The procedure was as follows: Flat glass slides were prepared with two paraffin rings. One drop of human red cell suspension plus 2 drops of undiluted rat serum were placed in each ring. The cells and serum were well mixed, first with a wooden applicator and then by rotating the slide. The suspensions were examined under the low power of a microscope for agglutination at intervals of 3, 5, 7, and 10 minutes at room temperature. Further mixing for 12–15 minutes showed little or no intensification of the reaction.

The degree of agglutination was recorded as trace, 1+, 2+, 3+, and 4+.

Trace: An occasional small clump, with a few loose, small aggregates of red blood cells in a mass of free-floating erythrocytes.

1+: A few cellular deforming clumps and a moderate num-

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[1] Originated from the King A inbred strain and further inbred for fifteen generations by P. M. Aptekman.

[2] Originated from the Wistar strain and inbred by M. R. Lewis.

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ber of loose aggregates of red blood cells among many free-floating erythrocytes.

2+: A moderate number of cellular deforming clumps with a few loose aggegates of red blood cells among many free-floating erythrocytes.

3+: Many cellular deforming clumps with a moderate number of free-floating erythrocytes.

4+: Complete agglutination, i.e., large cellular deforming clumps with none or just an occasional free-floating erythrocyte.

In calculating numerical titer averages, a trace of agglutination was given a value of 0.5; 1+ agglutination was given a value of 1.0; 2+ agglutination was given a value of 2.0, etc. It was found that the readings of a series of agglutination results compared to a later retesting of the sera showed a variation of ± 0.5 in the results.

RESULTS

Preliminary experiments on the P.A. strain of rats with various normal and tumor-bearing animals, and with suspensions of human erythrocytes from various blood groups, indicated that a standardization of the test for routine studies required the consideration of several problems, such as thermostability of the heteroagglutinins, the effect of pregnancy, and the relationship of age of the rat to heteroagglutinin titer.

The effect of various temperatures, for a period of 30 minutes, on agglutinin titer showed that these natural heteroagglutinins for human red cells, in rats of both the P.A. and Lewis strains, were thermostable at 55°–56° C., began to disappear at 58°–59° C., and were completely destroyed at 60°–61° C.

The influence of pregnancy on the titer of heteroagglutinins present in the serum of the P.A. rat produced no significant results when tested with erythrocytes from human blood of group AB. When tested with cells of the O blood group, the titers appeared somewhat lower during the last 2 or 3 days of gestation. To eliminate the influence of pregnancy in these experiments, the results from pregnant females were not included.

Studies on the relationship of the age of the host to the titer of natural heteroagglutinins indicated that antihuman erythrocyte agglutinin titer was low or entirely missing in very young rats, and the height of titer and the age of the rat were directly related. It became necessary, therefore, to ascertain the appearance of these natural agglutinins in reference to the age of normal rats before a study of the effect of tumor growth on agglutinin titer could be undertaken. As is illustrated in Chart 1, heteroagglutinins began to appear in low titer in rats of the P.A. strain when they reached 60–90 days of age. The titers varied from 0 to 2+ with red blood cells of group AB and from 0 to 1+ when tested with cells of the O group. Throughout the study the titer of heteroagglutinins for erythrocytes of group O blood was, on the average, lower than that for group AB blood. In rats 120 days of age and older, the titer of heteroagglutinins for AB cells was consistently higher, varying from 2+ to 4+, with an average of 3.2–4+. Heteroagglutinins for cells of group O lagged behind, and it was not until after the rats were 180 days old that a titer averaging 2.4+, with variations from 1+ to 4+, was maintained.

DISAPPEARANCE OF HEMAGGLUTININS FROM THE SERA OF TUMOR-BEARING RATS

Twelve mature, normal rats, of both sexes, and at an age when the highest agglutinin titers would be present (over 500 days old), were selected for

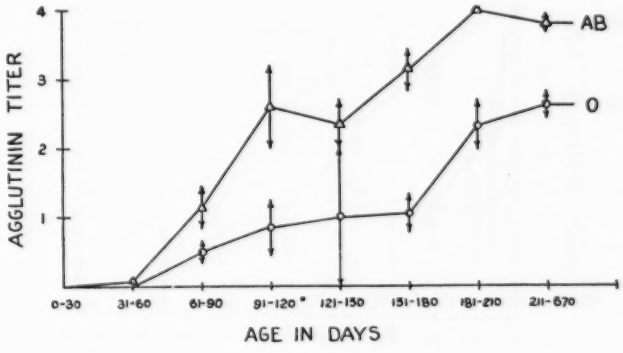

CHART 1.—Age of appearance of natural agglutinins for human group O and group AB blood cells in rats of the P.A. strain. The mean of five to 25 rats and the standard error found in the various age groups have been plotted. Standard error is calculated by $\sqrt{[\Sigma(\bar{x} - x)^2]/[n(n-1)]}$ and is represented by the vertical line through each point.

studies concerning the influence of growth of neoplastic tissue on the titer of hemagglutinins of the host. The rats were bled once to ascertain their normal heteroagglutinin titer. After this they were implanted with a portion (3-inch, No. 13-gauge trocal full) of viable tissue from Sarcoma 6. The titer of the hemagglutinins present in the sera, determined at nine different intervals (Chart 2), represents the mean of the results obtained from at least ten rats at each time of bleeding. Two rats of the original twelve died during the experiment.

Fifteen hours after implantation of the malignant tissue from Sarcoma 6, an appreciable drop in the titers of both AB and O blood cell groups occurred. This continued until the maximum immediate drop was reached in 48 hours. Thereafter, there was a rise in titer which reached a peak in 6–9 days. This rise, however, failed to reach the normal, pre-tumor implantation level. After 9 days there was a gradual and increasing drop in titer until the hemagglutinins disappeared entirely after 21 days (Chart 2). Normal controls with 4+ agglutinin titers maintained their 4+ titers

throughout the series of nine bleedings necessitated by this study.

In order to determine whether repeated bleeding of the rats (9 times in the first experiment) had any effect on the disappearance of the hemagglutinins, a second experiment was initiated with a larger number of rats. The animals (fifteen) were divided into three groups of five rats each. All the rats were bled once prior to implantation of tumor tissue, to determine the normal titer present in the serum of each rat. One group was then bled at 48 hours, another at 6 days, and the third group at 11 days after tumor implantation. All the rats were bled again at 21 days to determine agglutinin dis-

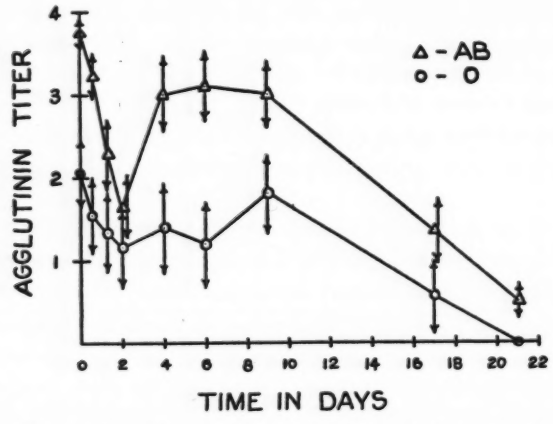

CHART 2.—Disappearance of hemagglutinins for human group O and group AB blood cells from the sera of P.A. rats after implantation of Sarcoma 6. Day 0 is the day of tumor implantation; the titers at this time were normal. The mean of ten rats at each determination and the standard error (vertical line through each point) are plotted.

appearance. Thus, the points of extreme fall and rise of titer were tested, and the titers given in Chart 3 represent the mean of the titers obtained from five rats at each time of bleeding. No rat was bled more than 3 times during the 21-day period.

This experiment bore out the first determination that, following tumor implantation, there is a drop in titer reaching a low level in 48 hours, then a rise (the mean of which fails to reach the pre-tumor implantation level), followed by a gradual fall in titer until a zero titer is reached after 21 days. This significant agreement indicates that repeated bleedings of 1 ml. did not lower the titer of heteroagglutinins present in the sera. During the progress of this second study, measurements of the tumors at each time of bleeding were made. The average size of the tumors, shown in Chart 3, indicates that an inverse relationship existed between hemagglutinin titer and tumor growth.

Hemagglutinin titer in normal and tumor-bearing rats of the Lewis strain.—Parallel studies carried out on the sera of normal rats of the Lewis strain showed that the titer of the heteroagglutinins for human red blood cells of the AB group was consistent and followed a pattern similar to that found in the normal rat of the P.A. strain. On the other hand, it was found that heteroagglutinins for red blood cells of the O group were missing in rats of the Lewis strain. Studies with Lewis rats also indi-

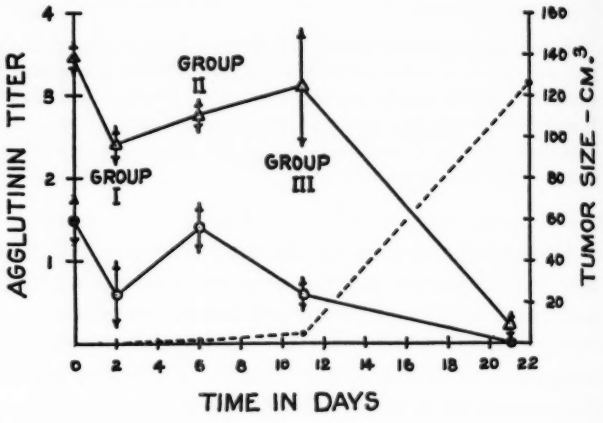

CHART 3.—Hemagglutinin titer for human group O (*circle*) and AB (*triangle*) blood cells in relation to tumor size in the P.A. strain. Day 0 is the day of tumor implantation; the titers at this time were normal. The mean of fifteen rats at day 0 and day 21, of five rats each at days 2, 6, and 9, and the standard error (vertical line through each point) are plotted. The dotted line represents growth of Sarcoma 6.

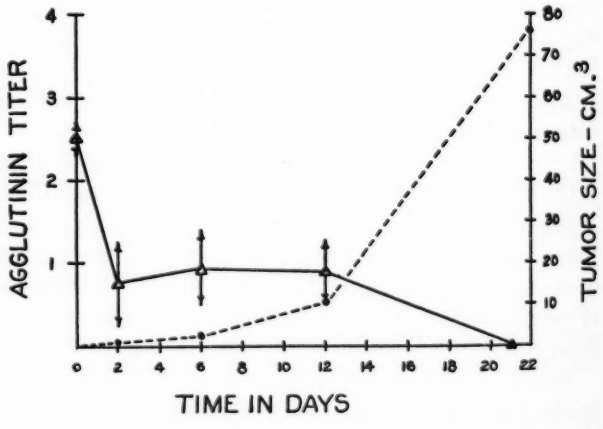

CHART 4.—Hemagglutinin titer for human group AB blood cells (*triangle*) in relation to tumor size in the Lewis strain. Day 0 is the day of tumor implantation; the titers at this time were normal. The mean of six rats at each determination and the standard error (vertical line through each point) are plotted. Serum from normal Lewis strain rats contains no natural agglutinins for human group O cells. The dotted line represents growth of Sarcoma 10.

cated that as many as nine bleedings did not influence the titer of the hemagglutinin.

The disappearance of hemagglutinins in relation to growth of Sarcoma 10 was investigated in rats of the Lewis strain. Each rat was bled 5 times (1 ml.), and the titer of each serum was deter-

mined. The titers recorded in Chart 4 represent the mean values from eight to ten rats at each time. The results illustrated in Charts 3 and 4 indicate that the systemic reactions to homologous tumor implantation and growth, as manifested by the anti-AB cell hemagglutinin titer, are consistent and follow a similar pattern in rats of the two highly inbred strains.

Influence of the various tumors on hemagglutinin titer.—It was important to ascertain whether the reactions of P.A. rats to Sarcoma 6 and of Lewis rats to Sarcoma 10 could be consistently obtained with certain other available homologous tumors. Thirty-eight rats (90–365 days old) bearing transplanted Sarcomas 231 and 6 and Carcinoma 5, native to the P.A. strain, and Sarcomas 3 and 10 and Lymphoma 8, native to the Lewis strain, were bled and the serum titers determined. In addition, several rats of each strain, bearing primary induced tumors, were tested. Animals with transplanted tumors were bled between 15 and 25 days after implantation of tumor grafts. Those with primary tumors were bled after the tumor had reached a moderately large size. The results are given in Table 1. They corroborate the previous experiments and are consistent for the rats of two different strains and for eight different native tumors.

TABLE 1

INFLUENCE OF VARIOUS TRANSPLANTED AND PRIMARY TUMORS ON HEMAGGLUTININ TITER

No. rats tested	Age (days)	Type of tumor	Tumor Size (Av. in c.cm.)	Agglutinin titer Group O	Group AB
		P.A. STRAIN			
3	90–100	Carcinoma 5	90	0	0
4	100–112	Sarcoma 231	90	0	0
8	107–110	Sarcoma 6	91	0	0
1	204	Primary*	101	0	0
6	90–120	Normal controls		trace	2+
3	181–210	Normal controls		2+	4+
		LEWIS STRAIN			
13	155–365	Sarcoma 10	76		0
4	255	Sarcoma 3	55		trace
3	160	Lymphoma 8	60		0
2	155	Primary*	68		0
5	155–160	Normal controls			2+
10	255–365	Normal controls			3+

* Induced by methylcholanthrene.

DISCUSSION

Evidence of other investigators (4, 5, 9, 11–13) points to an alteration of immunological response in human beings and animals bearing neoplastic growths. Parfentjev and his co-workers (11) found a decreased incidence of natural agglutinins for Proteus in patients with untreated neoplasms. Dubin (4), Wallhauser (13), and Howell (9) have reported significantly low titers of antibacterial antibody, as well as an associated impairment of immunological response, in patients with Hodgkin's disease and leukemia. Wharton and his co-workers (12) have shown that in mice with certain transplanted tumors the antisera had a decidedly lower titer than that from similarly treated normal mice. Goldman *et al.* (5) reported on a patient with a probable leukosarcoma in whom injections of horse serum effected only a minimal increase of sheep cell agglutinins.

Davidsohn and Stern (1–3) made extensive studies of natural heteroagglutinins for sheep cells in mice of low and high tumor strains. They found, however, no relationship between the occurrence of natural antisheep agglutinins and the incidence of spontaneous tumors in the individual strains. It is of interest to note the independence that appears to exist in this case among these natural heteroagglutinins. Whereas results from the present study of the tumor-bearing rat show the disappearance of natural agglutinins for human blood cells following tumor implantation and growth, Davidsohn and Stern (1) found that the presence of induced or transplanted tumors tended to raise the incidence and titer of antisheep agglutinins in mice. They also reported a lack of relationship between the presence and height of antisheep agglutinins and the presence and titer of antihuman blood cell agglutinins in mice.

Gorer (6, 7) reported the existence of antigenic differences in mice of inbred strains. He concluded that the presence of antigens, which were found in red blood cells and in mouse tumor tissue, was determined by the genetic constitution of the mice used. Lumsden (10) failed to find isoagglutinins in the sera of normal rats, but when spontaneous regression of an implanted alien strain tumor oc-

curred, isoagglutinins could be demonstrated in the sera of those rats. It would seem of value, therefore, to utilize animals of highly inbred strains bearing transplanted tumors that originated in the same inbred strain for this type of investigation. The results obtained with these rats showed less variation from the mean than would have been expected with a noninbred strain and heterologous tumors.

Since neither the P.A. strain nor the Lewis strain of rats has any appreciable incidence of spontaneous tumors, the immune response as reflected in antihuman erythrocyte agglutinin titers, determined in rats prior to implantation of viable tumor tissue, is assumed to be normal. It may be concluded, therefore, that the immediate drop in agglutinin titer followed by a rise in titer and ultimate disappearance which takes place following implantation of tumor tissue is primarily due to the presence of the tumor growing in the host.

SUMMARY

1. Natural heteroagglutinins for human red cells of Groups AB and O, in rats of both the P.A. and Lewis strains, were thermostable when heated at 55°–56° C. for 30 minutes but were completely destroyed when heated to 60°–61° C.

2. Trauma from repeated bleedings from the heart did not appear to affect the titer of heteroagglutinins in the sera.

3. The incidence and titer of natural heteroagglutinins in the sera of rats is related to the age of the rat. These agglutinins begin to appear in rats between the ages of 60 and 90 days, reaching consistently higher titers for human blood cells of groups O and AB after 180 days of age. Throughout this study the average titer for blood cells of group AB was higher than that for cells of group O blood. Sera from normal rats of the Lewis strain did not contain heteroagglutinins for cells of the O blood group. Otherwise, heteroagglutinin titers of of the sera from rats of the Lewis strain compared favorably with those from the P.A. strain.

4. Fifteen hours after implantation of malignant tissue in rats of either the P.A. or Lewis strain, an appreciable drop in the heteroagglutinin titers for cells of both O and AB blood groups occurred. The maximum immediate drop was reached in 48 hours. Thereafter, there was a rise in titers which reached a peak in 6–9 days. This rise, however, failed to reach the normal, pretumor implantation level. After 9 days there was a gradual and increasing drop in titer until the hemagglutinins disappeared entirely after 21 days.

5. Rats of both the P.A. and Lewis strains, with progressively growing native tumors, showed an inverse relationship between hemagglutinin titer and progressive tumor growth.

6. There was a consistent absence of hemagglutinins in the sera of 38 mature, tumor-bearing rats of the P.A. and Lewis strains. Each rat tested bore one of eight different transplantable tumors, or a primary induced tumor.

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The Effect of 2-Acetylaminofluorene on Growth and Composition of the Liver of the Rat*†

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In the first study of the toxicity and carcinogenic activity of 2-acetylaminofluorene (AAF), Wilson, DeEds, and Cox noted that the compound caused a profound inhibition of growth, when it was fed in a 10 per cent casein diet to albino rats (21). Inhibition of the growth of the rat has also been obtained with a number of carcinogenic aromatic hydrocarbons and with *p*-dimethylaminoazobenzene (11, 19, 20), and the growth-retarding ability of carcinogenic compounds has been considered a specific property of carcinogens inasmuch as structurally related noncarcinogenic compounds do not appear to possess this property (10). It is conceivable, therefore, that carcinogenic amines in producing growth inhibition cause the same or a similar metabolic disturbance as carcinogenic aromatic hydrocarbons and *p*-dimethylaminoazobenzene.

Inasmuch as increasing the level of dietary protein has been shown to protect against growth inhibition by 1,2,5,6-dibenzanthracene (DBA) (7), there is some reason to believe that carcinogenic aromatic hydrocarbons cause a disturbance of protein metabolism. Whether the carcinogen interferes with protein metabolism in general, or with the metabolism of specific amino acids only, is an unsettled problem. In the case of DBA, addition of cystine or methionine to a 5 per cent casein diet did not prevent the growth inhibition, indicating that DBA did not interfere with the metabolism of the sulfur amino acids (5). In contrast, either cystine or methionine was effective in relieving the growth inhibition induced by *p*-dimethylaminoazobenzene in rats maintained on a 6 per cent casein diet (19). In the case of 2-aminofluorene, recent work indicates that the carcinogen decreased the *in vitro* incorporation of methionine into rat liver protein (18). This suggests that carcinogenic amines may cause a depletion of sulfur amino acids in rat liver protein.

The present study was undertaken to show whether the growth inhibition induced in the rat by a tumor-producing concentration of AAF (16) is influenced by the level of dietary protein. In addition, the livers of pair-fed rats were analyzed to determine any changes in the nitrogen and sulfur content following prolonged intake of the carcinogen.

MATERIALS AND METHODS

Animals and diets.—Female rats, which have been reported to be more resistant to the toxic action of acetylaminofluorene than male rats (21), were used in the experiments. The animals of the Sprague-Dawley strain[1] had an average weight of 40 gm. and were 20–22 days old at the start of the experiment. The rats were caged individually in a room maintained at 26.5° C. and allowed water ad libitum. Food intake and body weight were recorded daily. The feeding jars were placed into tin cans (diameter, 8.4 cm.; height, 4.4 cm.) which were mounted in the center of 8-inch tin pie plates. This eliminated errors in the measurement of the food intake due to excessive spillage in the groups which consumed the carcinogen. Two basal diets, designated Diets A and B, were fed. The composition of Diet A was as follows: casein, 11; white corn dextrin, 66; sucrose, 16; salt mixture 12 (12), 4; corn oil, 2; cod liver oil, 0.05; inositol, 0.1; choline chloride, 0.2; liver concentrate (Wilson 1:20), 0.4. Diet B differed from Diet A only by containing 20 per cent casein and 57 per cent dextrin. Vitamins were added in mg/kg of diet as follows: thiamine hydrochloride, 5; riboflavin, 10; pyridoxine hydrochloride, 5; nicotinic acid, 5; calcium D-pantothenate, 25; *p*-aminobenzoic acid, 300; 2-methyl-1,4-naphthoquinone, 2; α-tocopherol, 210. Vitamin A was added to give 17,800 U.S.P. units/kg of food. In experiments 5 and 6 the rats were also given 2 drops of Navitol with Viosterol (Squibb) once weekly by mouth. 2-Acetylaminofluorene, m.p. 196°–198° C. (500 mg/kg of basal diet), was added in an acetone solution to the basal diets. The acetone was removed from the frequently stirred diets by a current of air. The control diets were treated with acetone alone in the same manner. The diets were stored in a cold room at 4° C.

[1] Obtained from the Holtzman Rat Company, Madison, Wis.

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Three different types of growth experiments were performed which are summarized in Table 1. In experiments 1 and 2 the rats were allowed food ad libitum. In experiments 3 and 4 pair-fed litter-mates were used, and the control rats were allowed as much of the basal diet as was consumed by their litter-mates which received the basal ration plus AAF. In experiments 5 and 6 groups of four litter-mates were used, and the food intake of all litter-mates in each group was restricted to that of the rat which received the 11 per cent casein ration plus AAF. This feeding method is referred to in Table 1 as double-paired.

Preparation of tissues and analytical methods.—At the termination of the growth experiments the rats were killed by neck stroke, the abdominal cavity and the chest were opened, and the animals were examined for the presence of grossly visible tumors. No tumors were found. The livers were removed, blotted, minced with scissors, and weighed. Water content was determined by drying the minced tissues at 103°–105° C. to constant weight. The dry tissues were ground to fine powders in a mortar, and the powders were extracted with diethyl ether in a Soxhlet apparatus for 24 hours. Total fat was determined by weighing the dry residue remaining after distillation of the ether. Total nitrogen was determined on 5–6-mg. aliquots of the fat-free liver powders by the micro-Kjeldahl procedure (15). Total sulfur was estimated gravimetrically as barium sulfate after oxidation of a suitable aliquot of the fat-free liver powders in a Parr bomb (2). The nitrogen and sulfur analyses were carried out in duplicate. In order to compare data obtained on rats of different weights, the analytical results, on a dry weight basis, were calculated and expressed as per cent of body weight. In all cases where the standard error was calculated, the statistical formulas applicable to small numbers of samples were used (1).

RESULTS

The effect of 0.05 per cent acetylaminofluorene incorporated into 11 and 20 per cent casein diets on the growth of the rat is shown in Table 1. When the rats were fed ad libitum, the addition of AAF to the 11 per cent casein diet produced a growth inhibition of 61 per cent. The growth inhibition was only 38 per cent when AAF was added to a 20 per cent casein ration. The extent of the growth retardation agrees with data of Wilson, DeEds, and Cox (21), who observed a 55 per cent growth inhibition when a 10 per cent casein diet containing 0.063 per cent AAF was fed ad libitum to female albino rats. Although in the above experiment the growth-inhibiting action of AAF appeared to be influenced by the casein level of the diet, evaluation of the data was complicated by the depressing effect which AAF had on food consumption. Table 1 shows that rats on AAF consumed only 40–50 per cent of the food which was eaten by the control rats.

TABLE 1

THE EFFECT OF 2-ACETYLAMINOFLUORENE ON THE GROWTH OF RATS ON 11 PER CENT AND 20 PER CENT CASEIN DIETS

Experiment no.*	Diet†	No. rats	Feeding method	Casein level (per cent)	Av. food intake/day/rat (gm.)	Av. initial wt.‡ (gm.)	Av. final wt.‡ (gm.)	Growth inhibition§ (per cent)
1C	Control	7	Ad libitum	11	12.7	43.2±1.7	193.4±6.9	
1E	AAF	7	"	11	6.1	42.7±1.4	100.9±4.6	61.0
2C	Control	7	"	20	12.0	43.7±1.5	201.1±5.8	
2E	AAF	7	"	20	7.5	40.2±0.9	138.3±6.3	37.6
3C	Control	8	Paired	11	5.7	40.5±0.9	102.8±2.6	
3E	AAF	8	"	11	5.7	42.7±0.8	84.4±2.4	33.1
4C	Control	8	"	20	7.5	47.6±1.0	138.5±5.0	
4E	AAF	8	"	20	7.5	43.0±0.5	135.2±3.5	0.0
5C	Control	7	Double-paired#	11	5.3	35.1±0.8	104.5±3.8	
5E	AAF	7	"	11	5.3	35.2±0.7	93.2±3.6	16.5
6C	Control	5‖	"	20	5.5**	35.1±1.1	121.9±7.7	
6E	AAF	5‖	"	20	5.5	36.3±1.3	122.6±6.4	0.0

* Experiments 1 and 2 were carried out for 75 days, experiments 3 and 4 for 82 days, and experiments 5 and 6 for 69 days.

† AAF was added to the basal diets to give a concentration of 0.05 per cent.

‡ Average weight ± standard error of the mean.

§ Growth inhibition $= \frac{\text{Weight gain of control rats} - \text{weight gain of treated rats}}{\text{Weight gain of control rats}} \times 100.$

See text.

‖ Two rats died accidentally before termination of the experiment.

** This average is slightly higher than 5.3 gm/day, since the two rats which had died prior to the termination of the experiment belonged to groups of litter-mates which consumed only 5 gm/day.

The experiments were therefore repeated on pair-fed rats. Although under these conditions the food intake of the rats which received AAF and that of the controls was the same, Table 1 shows that the food consumption of rats maintained on the 20 per cent casein diet plus AAF was approximately 35 per cent greater than that of rats fed the 11 per cent casein diet plus AAF. The carcinogen inhibited growth on the 11 per cent casein diet to the extent of 33 per cent, but had no effect on the growth of rats maintained on the 20 per cent casein ration, even though these animals consumed larger amounts of AAF. In experiments 5 and 6, com-

parable food and carcinogen intake on both diets was insured by the feeding method described above. Under these conditions, AAF retarded the growth of rats on the 11 per cent casein ration to the extent of 17 per cent. As in experiment 4, there was no growth inhibition on the 20 per cent casein ration. These experiments demonstrate conclusively, therefore, that the level of dietary protein determines whether AAF acts as a growth inhibitor for the rat. It will also be seen that the growth retardation due to AAF is not so extensive as was anticipated as a result of the ad libitum experiments if precautions were taken to insure equal food intake.

The effect of AAF on liver size and liver composition is summarized in Tables 2 and 3. Table 2 shows that AAF increased the ratio of liver to body weight using either ad libitum or paired feeding methods. In experiment 1 the ratio of dry liver to body weight increased 47 per cent and in experiment 2, 36 per cent. In experiments 3 and 4, the increases were 33 and 79 per cent, respectively. These results confirm previous reports of liver enlargement after prolonged intake of AAF, both in adult and in growing rats which were fed ad libitum (9, 14). Similar effects on liver size have been observed in mice and rats treated with polycyclic and heterocyclic carcinogenic compounds which were not aromatic amines (4, 6, 13). Enlargement of the liver despite subnormal body growth appears to be a common effect of a relatively large variety of carcinogens not closely related in structure.

The effect of AAF on liver fat, nitrogen, and sulfur is shown in Table 3. AAF had no effect on the percentage of fat, whereas the percentages of nitrogen and sulfur were decreased on both diets to approximately the same degree. However, the total quantities of fat, nitrogen, and sulfur, when expressed in per cent of body weight, were found to be significantly greater in the livers of rats which were fed AAF. The constant sulfur to nitrogen ratios in all groups indicate that there was no depletion *in vivo* of the sulfur amino acids due to AAF. The last column of Table 3 shows that part of the liver dry weight which was not accounted for by either protein or fat. It appears that this fraction, which undoubtedly consists of carbohydrate, was markedly increased (four to tenfold) in livers of rats consuming AAF. This suggests that AAF may stimulate deposition of liver glycogen. This point is being further investigated. Table 4 shows that the increase in liver size for a given AAF concentration in the diet is greater on a 20

TABLE 2

THE EFFECT OF 2-ACETYLAMINOFLUORENE ON RAT LIVER SIZE*

Exp. no.	Diet†	Wet weight of liver (gm.)	Water (per cent)	Dry weight of liver (gm.)	Wet weight/ 100 gm. body wt.	Dry weight/ 100 gm. body wt.
1C	Control	5.65±0.34	71.4±1.1	1.61±0.09	2.92±0.12	0.83±0.03
1E	AAF	4.35±0.33	70.9±0.7	1.24±0.11	4.20±0.16	1.22±0.14
2C	Control	5.12±0.13	70.6±0.2	1.50±0.04	2.54±0.05	0.75±0.02
2E	AAF	5.03±0.33	71.7±0.3	1.42±0.09	3.73±0.12	1.02±0.04
3C	Control	3.08±0.18	71.5±0.4	0.88±0.03	2.99±0.07	0.85±0.02
3E	AAF	3.27±0.14	70.5±0.3	0.96±0.02	3.87±0.04	1.13±0.03
4C	Control	3.54±0.18	70.2±0.4	1.05±0.04	2.56±0.09	0.76±0.01
4E	AAF	6.33±0.31	70.9±0.4	1.84±0.10	4.72±0.17	1.36±0.05

* All data are arithmetic means ± standard error of the mean.
† AAF was added to the basal diets to give a concentration of 0.05 per cent.

TABLE 3

THE EFFECT OF 2-ACETYLAMINOFLUORENE ON THE COMPOSITION OF RAT LIVER*

Exp. no.	Diet†	Total fat (per cent)	Nitrogen (per cent)	Sulfur (per cent)	Total fat/ 100 gm BWT (Mg.)‡	Total nitrogen/ 100 gm BWT (Mg.)	Total sulfur/ 100 gm BWT (Mg.)	Total S/ Total N	Residue§/100 gm BWT (Mg.)
3C	Control	12.6±0.37	14.8±0.05	0.94±0.03	104 ±4	109±3	6.9±0.5	0.063	63± 8
3E	AAF	13.4±0.64	12.2±0.05	0.75±0.01	151 ±6	120±2	7.4±0.8	0.062	230±22
4C	Control	13.1±0.78	15.5±0.08	1.10±0.03	95.2±4	103±3	7.5±0.8	0.073	25± 4
4E	AAF	12.5±0.44	12.6±0.06	0.83±0.01	188 ±9	142±6	9.8±0.8	0.069	263± 9

* The data are arithmetic means ± standard error of the mean.
† AAF was added to the basal diets to give a concentration of 0.05 per cent.
‡ BWT = body weight.
§ Mg. residue = Mg. dry weight − (mg. total nitrogen × 6.25 + mg. total fat).

per cent casein ration than on an 11 per cent casein diet. In addition, it will be seen that on the high casein ration protein contributed to the liver enlargement to a greater extent than fat or carbohydrate. As had been noted in the growth experiments, utilization of dietary protein in the presence of AAF was, therefore, better on a 20 per cent than on an 11 per cent casein ration.

DISCUSSION

The present experiments allow the conclusion that the action of AAF as a growth inhibitor for the rat depends on the level of dietary protein. However, even on a low protein diet AAF is not so powerful in retarding growth as might appear from earlier work (21). The present study shows that control of food intake is essential for the evaluation of a carcinogen as a growth inhibitor.

TABLE 4

EFFECT OF THE LEVEL OF CASEIN ON THE CHANGES IN RAT LIVER COMPOSITION INDUCED BY 0.05 PER CENT AAF IN PAIR-FED RATS*

Exp. no.	Diet	Δ dry weight†/ 100 gm body weight	Δ total fat†/ 100 gm body weight	Δ total protein†/ 100 gm body weight	Δ residue†/ 100 gm body weight
3	11 per cent casein	280±25	47± 7	70±22	167±22
4	20 per cent casein	600±51	93±10	244±39	238±26

* All values are differences of arithmetic means ± standard error of the mean.

† Δ/100 gm body weight = $\frac{\text{Mg. tissue or tissue component of AAF-treated rat}}{\text{100 gm. body weight}} - \frac{\text{Mg. tissue or tissue component of control rat}}{\text{100 gm. body weight}}$.

The data of Elson and Warren obtained with DBA (7) and the present experiments with AAF demonstrate that both carcinogens, when administered by routes and in quantities which will produce tumors, exert a growth retardation which is manifest only on diets which are relatively low in protein. It appears likely from these observations that carcinogenic polycyclic hydrocarbons and carcinogenic amines cause disturbances of growth by similar mechanisms, and the available evidence indicates that both types of carcinogens interfere in some way with protein metabolism.

It might be inferred from the work of Wase, Allison, and Migliarese (18) that AAF has a specific effect on the metabolism of methionine and thus retards growth. However, if AAF interfered only with the metabolism of methionine, one would hardly expect that a 20 per cent casein diet, which supplies just enough methionine for growth (3, 17), would be effective in protecting against the growth-inhibiting action of AAF. Further experiments are necessary to determine whether the action of AAF on growth involves specifically the metabolism of methionine.

Although AAF has no effect on growth on a 20 per cent casein ration, the incidence of mammary, ear duct, and liver tumors at this level of casein is very high and as great as on a 9 per cent casein diet (8). This is of interest since it has been postulated that there is a causal relationship between the growth-inhibitory properties of carcinogenic compounds and their ability to give rise to tumors (10). The present evidence, in conjunction with the data of Engel and Copeland on tumor production by AAF cited above, does not support this view. In the case of AAF, it would appear doubtful that carcinogenic activity and the ability to retard growth are etiologically related.

SUMMARY

1. The effect of 11 per cent and 20 per cent casein diets on the inhibitory action of AAF on the growth of the rat was studied. Using pair-fed rats, it was found that AAF inhibits growth on the 11 per cent but not on the 20 per cent casein ration.

2. AAF caused enlargement of the liver on both diets. Determinations of the fat, nitrogen, and sulfur content of the liver indicated that the composition of the livers of rats which had consumed AAF differed significantly from the composition of the livers of normal rats. There was no evidence for a sulfur depletion of the livers of rats consuming AAF.

3. The significance of the results is discussed and compared to similar data obtained with DBA.

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